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Photoswitching of peroxidase activity by position-specific incorporation of a photoisomerizable non-natural amino acid into horseradish peroxidase

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Abstract Horseradish peroxidase mutants containing L-*p*-phenylazophenylalanine (azoAla) at various positions were synthesized by using an *Escherichia coli* in vitro translation system. Among the 15 mutants examined, four mutants containing a single azoAla unit at the 6th, 68th, 142nd, and 179th positions, respectively, retained the peroxidase activity. The activity of the Phe68azoAla mutant was higher when the azobenzene group was in the *cis* form than in the *trans* form. On the contrary, the activity of the Phe179azoAla mutant disappeared when the azobenzene group was photoisomerized to the *cis* form, but recovered in the *trans* form. In the latter mutant, therefore, an on/off photoswitching of the peroxidase activity was attained.

Key words: Photoswitching; Non-natural mutagenesis; Four-base codon; Phenylazophenylalanine; In vitro translation

1. Introduction

Switching of enzyme activity by external signals, such as photon, has been attracting interest as a technique for controlling biochemical reactions in vitro and in vivo. The photoswitching has been achieved by introducing photochromic groups into proteins [1–6]. For example, Willner et al. [1] reported that the activity of papain could be photocontrolled by the *cis*/*trans* photoisomerization of an azobenzene group that were chemically linked to Lys residues. Liu et al. [5] and Hamachi et al. [6] reported photocontrol of RNase S activity that contained chemically synthesized S-peptide incorporated with a *p*-phenylazophenylalanine (azoAla).

We have been developing a four-base codon strategy for incorporating non-natural amino acids into proteins and found that an azoAla unit could be introduced into proteins with reasonable efficiency [7,8]. In this study, we have synthesized horseradish peroxidase (HRP) mutants that contain a single azoAla unit at various positions, using an *Escherichia coli* in vitro translation system where the position of the azoAla unit was directed by a CGGG four-base codon. The activities of the HRP mutants were evaluated under different photoirradiation conditions that lead to *trans*- and *cis*-rich azobenzene groups.

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2. Materials and methods

2.1. Preparation of $tRNA_{CCCG}$ carrying an azoAla

A tRNA that contained a CCCG anti-codon and was aminoacylated with azoAla was synthesized as previously described [7,8]. A tRNA fragment that contained a CCCG anti-codon and lacked a CA dinucleotide unit at the 3′-terminal was transcripted by using T7 RNA polymerase. azoAla was synthesized according to the literature [9], and linked with a pdCpA dinucleotide. Then, the azoAla–pdCpA was ligated with the $tRNA_{CCCG}$(-CA) by T4 RNA ligase.

2.2. Preparation of mRNA encoding HRP

A plasmid encoding HRP was mutated to replace an AAC codon at the position 255 by a GAC codon to introduce an N255D mutation. A CGG codon at the position 224 was also replaced by a CGT codon, in order to use a CGGG four-base codon. The resulting gene was cloned into the T7 expression vector [7] containing a T7 tag at the N-terminal. HRP mutant genes containing a CGGG four-base codon at the Phe6, Phe45, Phe68, Asn72, Ser73, Leu138, Ala140, Phe142, Phe152, Gly156, Leu163, Phe172, Phe179, Tyr185, and Leu205 positions, respectively, were prepared by the PCR. Then, the corresponding mRNAs were prepared by using T7 RNA polymerase.

2.3. In vitro translation of mutant HRP

The azoAla-$tRNA_{CCCG}$ and the mRNA were added to an *E. coli* in vitro translation system. The translation was carried out in 10 μl of the reaction mixture containing 55 mM HEPES–KOH, pH 7.5, 210 mM potassium glutamate, 6.9 mM ammonium acetate, 12 mM magnesium acetate, 1.7 mM DTT, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethyleneglycol-8000, 35 μg/ml folinic acid, 0.1 mM each amino acid, 16 μg mRNA, and 2 μl of *E. coli* S-30 extract. The reaction mixture was incubated at 37°C for 60 min and subjected to 15% SDS–PAGE. The Western blotting was carried out by using anti-T7 tag antibody (Novagen) and alkaline phosphatase-labelled anti-mouse IgG (Promega).

2.4. Refolding of the engineered HRPs and evaluation of the peroxidase activity

Urea was added to the translation mixture to give the final concentration of 6 M and the resulting solution was incubated at 30°C for 1 h, then at 0°C for 1 h. The solution was then diluted to 1/10 with a refolding medium (19.4 mM Tris–HCl, pH 8.0, 0.94 mM EDTA, 1.6 M urea, 5.5 mM $CaCl_2$, 0.77 mM oxidized glutathione) and incubated at 20°C. The refolding mixture was gel-filtrated by using Micro Bio-Spin P-6 column (Bio-Rad).

A substrate solution (100 μl) containing 50 mM disodium phosphate, 25 mM citric acid, pH 5.0, 0.4 mg/ml *o*-phenylenediamine, and 0.007% hydrogen peroxide, was added to 25 μl of the elute solution on a 96-well microplate. The absorbance at 450 nm was followed on a microplate reader (Bio-Rad) as a measure of the peroxidase activity.

The effect of hemin addition into the refolding mixture of the wild-type HRP was examined over the hemin concentrations from 0 to 10 μM. However, the peroxidase activity was highest in the absence of extra hemin. This suggests that the *E. coli* S-30 extract contains sufficient amount of heme group that may bind to the HRP synthesized.

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3. Results and discussion

3.1. Expression of HRP mutants containing azoAla

The N255D mutation has been reported to enhance correct refolding [10]. The N255D mutant was linked with a T7 tag at the N-terminal for efficient translation in the *E. coli* S-30 system and for easy detection by anti-T7 tag antibody. The resulting N255D mutant with a T7 tag will be called as a wild-type HRP hereafter.

Single azoAla was introduced at the Phe6, Phe45, Phe68, Asn72, Ser73, Leu138, Ala140, Phe142, Phe152, Gly156, Leu163, Phe172, Phe179, Tyr185, and Leu205 positions, respectively. The expression of each mutant in the in vitro system was confirmed on Western blotting using anti-T7 tag antibody. In the presence of azoAla-tRNA$_{CCCG}$, full-length HRPs were observed at 34 kDa in all cases (Fig. 1). On the other hand, only truncated proteins were detected in the absence of the azoAla-tRNA$_{CCCG}$. The results indicate that azoAla has been successfully incorporated into respective positions.

3.2. Refolding and selection of the mutants with peroxidase activity

The wild-type and the mutant HRPs showed little peroxidase activity just after the in vitro synthesis, probably due to an insufficient and/or incorrect folding. As described in the experimental section, the refolding was carried out according to the procedure reported by Smith et al. [11]. The wild-type HRP after the refolding process showed the peroxidase activity that corresponded to 0.01 μg/ml of native HRP. The Phe6, Phe68, Phe142, and Phe179 mutants showed the peroxidase activity that corresponds to 3–10% of the wild-type activity. In comparing these peroxidase activities, the observed absorbance has been normalized by the protein concentration that was evaluated from the band intensity of the Western blotting. Other 11 mutants showed even smaller activities (Fig. 1). The absence of the peroxidase activity in the majority of the mutants indicates that the introduction of an azoAla unit is unfavorable for the folding of HRP, due to a large-sized and highly hydrophobic azobenzene group.

Because all the reaction mixtures prepared in the absence of azoAla-tRNA$_{CCCG}$ showed no peroxidase activity, the truncated proteins must be inactive.

3.3. Photocontrol of the peroxidase activity

The effect of photoisomerization of the azobenzene group on the peroxidase activity was examined. The product of the in vitro synthesis, followed by the refolding process, was irradiated for 1 min by a 150 W Xe lamp passed through a grating monochrometer at 350 ± 10 nm for UV irradiation and at 460 ± 10 nm for visible irradiation. Under these conditions, a monomeric azoAla unit reached up to 90% *cis* form after UV irradiation and 70% *trans* form after visible irradiation. Although longer visible irradiation led to higher percentage of the *trans* form, it also caused an irreversible damage of the HRP activity. Time courses of the enzyme reactions just after UV or visible irradiation are shown in Fig. 2. Under the present conditions, peroxide activity of the wild-type HRP was insensitive to the UV or visible irradiation (Fig. 2A). On the other hand, the activity of the Phe68azoAla mutant markedly increased after UV irradiation and decreased after visible irradiation. The change of the peroxidase activity could be repeated several times and the enzyme activity was solely determined by the final irradiation wavelengths. The photocontrol can be interpreted in terms of a reversible photoisomeri-

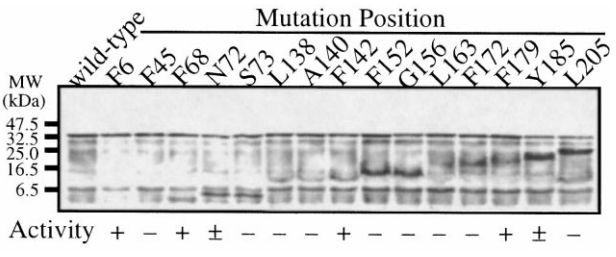

Fig. 1. Western blot analyses of the translation products of the *E. coli* in vitro translation systems. Each translation mixture contains a mRNA with a CGGG four-base codon at a specific position and a phenylazophenylalanyl-tRNA with a CCCG four-base codon. The signs under the figure indicate peroxidase activities normalized by the protein concentrations: +, 3–10% with respect to the wild-type; ±, smaller than about 3% but detectable activity; −, no activity.

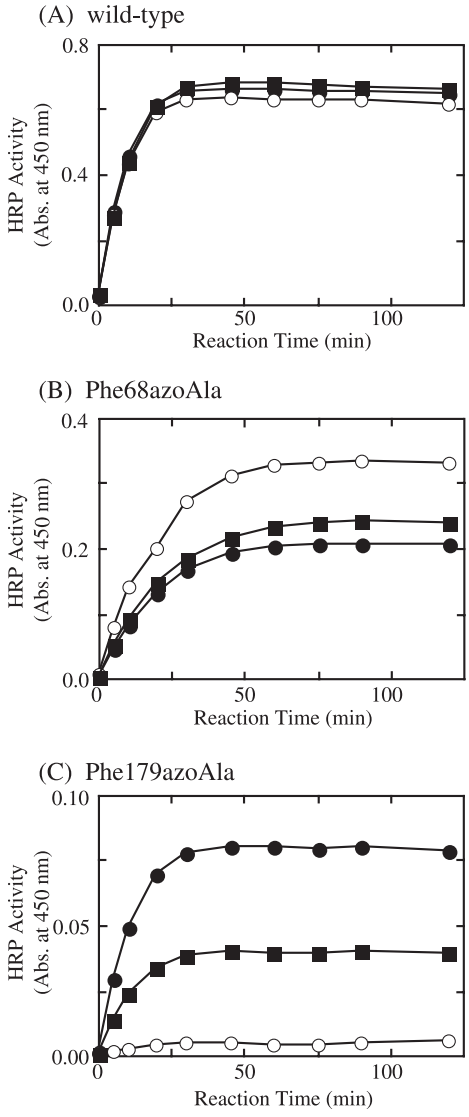

Fig. 2. Time courses of the oxidation of *o*-phenylenediamine by the wild-type HRP (N255D) (A), by Phe68azoAla mutant (B), and by Phe179azoAla mutant (C) under alternating photoirradiations before the enzyme reaction: no irradiation (solid circles), UV irradiation (open circles), and UV→visible irradiation (solid squares).

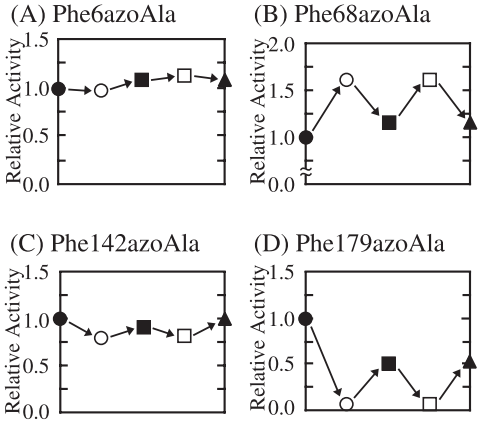

Fig. 3. Relative peroxidase activities after alternating UV and visible irradiations. Each sample was irradiated on the following sequences: no irradiation (solid circles), UV irradiation (open circles), UV→visible irradiation (solid squares), UV→visible→UV irradiation (open squares), UV→visible→UV→visible irradiation (solid triangles). The peroxidase activities were evaluated by OD_{450} after 120 min.

zation of the azobenzene group. Since the 68th position is close to the substrate-binding site [12], the photoisomerization of azobenzene group would affect the substrate binding.

Contrary to the Phe68azoAla case, the activity of the Phe179azoAla mutant almost disappeared after UV irradiation, but recovered by visible irradiation. Again, the photocontrol could be repeated many times. The results indicate that, contrary to the case of Phe68azoAla, the Phe179azoAla mutant becomes inactive when the azobenzene group changed to the *cis* form, but recovers the activity when the *trans* form was resumed. The 179th position is also located near the substrate-binding site, but the isomerization of the azobenzene group at this position affects the substrate binding in a different way. The opposite effect suggests that the photocontrol is not based on the environmental change of the polarization by the *cis*/*trans* photoisomerization, but based on a side-chain conformational change that affects the substrate binding or the entire protein conformation. The incomplete recovery of the peroxidase activity after visible irradiation may be due to an incomplete recovery of the *trans* form as described above.

The peroxidase activities of the above and other mutants after UV and visible irradiation are plotted in Fig. 3. The Phe142azoAla mutant was relatively insensitive to the irradiation, although the 142nd position is close to the substrate-binding site. The insensitiveness of the Phe6azoAla mutant may be reasonably explained in terms of the distance of the 6th position to the binding site. Other mutants that are not included in Fig. 3 showed peroxidase activity neither after UV irradiation nor after visible irradiation.

The above results proved that the success of the photocontrol of enzyme activity by an azobenzene group depends sensitively on the incorporation positions. The enzyme activity will be increased, decreased, or unaffected by the *trans* to *cis* photoisomerization, depending on the positions of the azobenzene group. To achieve a sharp photoresponsibility, position-specific incorporation of azoAla or other photoisomerizable non-natural amino acids will be essential.

3.4. Conclusion

HRP mutants that contain an azoAla unit at various positions were synthesized in an *E. coli* in vitro translation system by using the four-base codon strategy. The peroxidase activities of the Phe68azoAla and Phe179azoAla mutants were determined by the final wavelengths after alternating photoirradiation with UV and visible light. An on/off type photoswitching was achieved for the Phe179azoAla mutant with 'on' in the *trans* form and 'off' in the *cis* form. It must be stressed that the sharpness and the direction of the photoswitching depend on a proper selection of the position of the azobenzene group. In this sense, the position-specific incorporation of an azoAla unit through the four-base codon strategy is a general and promising strategy to obtain photoswitchable proteins.

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References

[1] Willner, I., Rubin, S. and Riklin, A. (1991) J. Am. Chem. Soc. 113, 3321–3325.
[2] Willner, I., Rubin, S. and Zor, T. (1991) J. Am. Chem. Soc. 113, 4013–4014.
[3] Westmark, P.R., Kelly, J.P. and Smith, B.D. (1993) J. Am. Chem. Soc. 115, 3416–3419.
[4] Hohsaka, T., Kawashima, K. and Sisido, M. (1994) J. Am. Chem. Soc. 116, 413–414.
[5] Liu, D., Karanicolas, J., Yu, C., Zhang, Z. and Woolley, G.A. (1997) Bioorg. Med. Chem. Lett. 7, 2677–2680.
[6] Hamachi, I., Hiraoka, T., Yamada, Y. and Shinkai, S. (1998) Chem. Lett., 537–538.
[7] Hohsaka, T., Ashizuka, Y., Murakami, H. and Sisido, M. (1996) J. Am. Chem. Soc. 118, 9778–9779.
[8] Hohsaka, T., Kajihara, D., Ashizuka, Y., Murakami, H. and Sisido, M. (1999) J. Am. Chem. Soc. 121, 34–40.
[9] Goodman, M. and Kossoy, A. (1966) J. Am. Chem. Soc. 88, 5010–5015.
[10] Lin, Z., Thorsen, T. and Arnold, F.H. (1999) Biotechnol. Pros. 15, 467–471.
[11] Smith, A.T., Santama, N., Dacey, S., Edwards, M., Bray, R.C., Thorneley, R.N.F. and Burke, J.F. (1990) J. Biol. Chem. 265, 13335–13343.
[12] Gajhede, M., Schuller, D.J., Henriksen, A., Smith, A.T. and Poulos, T.L. (1997) Nat. Struct. Biol. 4, 1032–1038.