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Modulation of the catalytic mechanism of hen egg white lysozyme (HEWL) by photochromism of azobenzene

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Abstract

Hen egg white lysozyme (HEWL) (EC3.2.1.17) was modified with azobenzene (AZB) and the influences of photochromism of AZB on the enzyme activity was studied. Mass spectrometry (MALDI-TOF MS) study revealed that one molecule of AZB-4-carboxylic acid binds to ε-amino group of Lys33 located in the back space of the substrate binding site F, through amide bond. The reversible photochromism of AZB in HEWL was confirmed by spectrophotometric measurement. The irradiation with UV light to *trans*-AZB HEWL (AZB*t*-L) generated two types of *cis*-AZB HEWL (AZB*c*-L) which were named as AZB*c*l-L and AZB*c*2-L in elution order on a reversed phase HPLC. The fraction at the photostationary state (366 nm) was consisted of AZB*t*-L (20%), AZB*c*l-L (50%), and AZB*c*2-L (30%). Catalytic efficiencies (k_{cat}/K_m) of AZB-Ls lowered to one-half of the native enzyme efficiency. Maximum initial rate (V_{max}) and Michaelis–Menten constant (K_m) of AZB*t*-L were 1.50×10^{-1} mg ml^{-1} s^{-1}, 3.00×10^{-1} mg ml^{-1}, respectively. The fraction of AZB*c*-Ls showed 1.05×10^{-1} of V_{max} and 1.82×10^{-1} of K_m. The isomerization from *trans*-form to *cis*-form accompanied the decrease of V_{max} and the increase of the affinity for substrate (K_m). ^{1}H NMR study suggests that the modulation may be caused by the configurational change of Glu35 via Trpl08 induced by the photochromism of AZB.

Keywords: Photochromism; Hen egg white lysozyme; Azobenzene

1. Introduction

Until now, a large number of studies to control enzyme activities by photochromism have been reported [1–13]. The change of enzyme activity according to the photochromism has been investigated well. In many of those studies, however, the influences of the chemical modification of the enzymes with the photochromic compounds and of the photochromism on the substantial changes of the enzymes in themselves have not been discussed in detail. The enzyme reaction consists of two main steps, the formation of enzyme–substrate complex and the following catalytic action to produce the reaction products. In the process, catalytic activity is induced by the conformational and/or configurational changes at the enzyme active center initiated by the binding of substrate. It is well known that, in many cases, the chemical modification changes local and/or whole structures of proteins and makes the inherent biochemical and physicochemical properties alter. Such a method has contributed to make the catalytic mechanisms of enzymes clear.

This study was performed from a point of view that the major factors, which exert the influences on the change of enzyme activity are not only the photochromism of but also the attachment of photochromic compound. In this study,

Abbreviations: THF, tetrahydrofuran; DCC, *N*,*N*′-dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; NHS, *N*-hydroxysuccinimide; TCEP, tris-(2-carboxyethyl) phosphine; Sinapinic acid, 3,5-dimethoxy-4-hydroxycinnamic acid; CHCA, α-cyano-4-hydroxycinnamic acid; AAEE, acyloyl-aminoethoxyethanol

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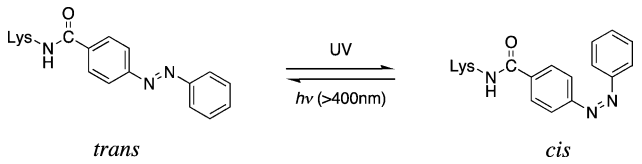

Fig. 1. Photochromism of 4-(phenylazo) benzoic acid introduced into lysine residue through amide bond.

azobenzene (AZB) is used as a photochromic compound which shows the typical photoreaction (Fig. 1). Hen egg white lysozyme (HEWL) (EC3.2.1.17) in which molecule ε-amino group of lysine only at residue33 was amidated with azobenzene-4-carboxylic acid was prepared. Based on the results of the following experiments, measurements of p*I* values, the absorption spectra and fluorescent intensity, and analyses of the chromatographic behaviors on a cation exchange and reversed phase HPLC and ^{1}H-one and two-dimensional NMR spectra of native, *cis*-AZB- and *trans*-AZB-HEWLs, we could draw the conclusion that the enzyme activity of HEWL was modulated by the phtochromism of the attached AZB molecule.

2. Materials and methods

2.1. Photochromic lysozyme

Modification of lysine residues in HEWL with 4-(phenylazo) benzoic acid was carried out according to the method described by Willner et al. [12].

A cold THF solution (5 ml) containing 4-(phenylazo) benzoic acid (0.23 g, 1 mmol) and NHS (0.12 g, 1 mmol) was added to 3 ml of cold THF solution containing DCC (0.21 g, 1 mmol). The mixture was stirred for 2 h at 0 °C, and left overnight at 4 °C. The resulting active ester solution was filtered into cooled aqueous solution (45 ml) containing sodium dicarbonate (2.0 g) and HEWL (2.0 g, 0.14 mmol). The mixture was stirred for 6 h at 0 °C, left overnight at 4 °C and extracted with ethyl acetate to remove unreacted active ester. The water phase, which contained the crude AZB-L, was desalted by dialysis against distilled water at 4 °C and then lyophilized. The lyophilized powder was dissolved in 10 ml of 10 mM Tris–buffer (pH 7.0) and loaded on a CM-Sepharose FF (Na^+ form, Amersham Biosciences, Uppsala, Sweden) column (2.4 cm × 30 cm) equilibrated with the same buffer. The column was washed with five volumes of 10 mM Tris–buffer (pH 7.0) and then the proteins were eluted with linear gradient from 0.1 to 1.0 M of NaCl in the buffer. The elution profile was monitored at 280 nm. The fraction which contained AZB-L was checked at 350 nm. The main fraction at 350 nm was dialyzed against distilled water at 4 °C and then lyophilized.

Further purification was carried out on a HPLC (CCPM-II, TOSOH, Tokyo, Japan) equipped with an ODS column (SG300, C18, 5 μm, 4.6 mm × 250 mm, Shiseido Fine chemicals, Tokyo, Japan), monitoring with a photodiodearrey detector (PD-8020, TOSOH). As mobile phases, eluent A (0.1% TFA in water) and eluent B (0.1% TFA in 70% acetonitrile/30% water by volume) were used. Proteins were separated by isocratic elution with 40% of the eluent B for 15 min, followed by linear gradient elution from 40 to 80% of the eluent B for 40 min. The flow rate was 1 ml/min. By this method, AZB-L was prepared as AZB*t*-L in the purity of more than 98%.

2.2. Preparation of AZBt-Land AZBc-Ls

Preparation of the *cis*-form compounds was achieved by irradiation to the *trans*-form with UV light. The UV light source was 200 W high pressure mercury lamp (UHD-200DP, USHIO, Tokyo) from which the light at 366 nm was isolated by filters (aqueous solution, Toshiba UV-D35 and UV35 glass filters). The visible light source to turn back the *cis* form to the *trans* form was 150 W xenon lamp (L-2175, Hamamatsu), equipped with filters (aqueous solution, HOYA Y42).

2.3. Isoelectric focusing

Isoelectric focusing (IEF) was performed on a BioFocus 3000 capillary electrophoretic system (Bio-Rad Laboratories, CA) with an AAEE-coated capillary (24 cm × 50 μm), monitoring at 280 and 340 nm. The calibration of isoelectric point (p*I*) was performed using synthetic p*I* markers (BioMark p*I* marker set, Bio-Rad Laboratories).

2.4. Digestion with trypsin

Protein was dissolved in 0.01 M $(NH_4)_2CO_3$ buffer (pH 8.1) at a concentration of 2 mg/ml. Into 50 μl of the protein solution, 1 μl of trypsin (Sigma–Aldrich, St. Louis, MS) solution (1 mg/500 μl H_2O) was added. Digestion was carried out at 37 °C with an enzyme/substrate ratio of 1:50. After the incubation for 5 h, the reaction mixture was divided into two portions. Into one portion, 1 μl of TCEP solution (1.9 mg/190 μl H_2O) was added and the mixture was allowed to stand at room temperature for 10 min to reduce disulfide bond. The reaction of each portion was stopped by the addition of 1 μl of 10% TFA.

2.5. Mass analysis

Mass analyses of proteins and tryptic peptides with MALDI-TOF MS were performed on a Voyager-DE STR (PE Biosystems, Framingham, MA). Mass spectra of the samples were acquired using sinapinic acid and CHCA as matrices.

2.6. CD-spectrometry

CD spectra of proteins in the range of 190–240 nm were obtained on a J-720 Spectropolarimeter (JASCO, Tokyo, Japan) using 1 and 5 mm cells.

2.7. NMR experiments

For the NMR measurements, samples were prepared by dissolving native or AZB-modified lysozymes in 500 μl of water containing 10% D_2O. The final protein concentration of the solution was 2 mM. The pH of the sample solution was adjusted to 2.2. NMR experiments were carried out in dark at 25 °C on a Bruker DMX-500 spectrometer. Two-dimensional NMR experiments including NOESY [14] and ^1H–^{13}C HSQC [15] experiments were carried out using the WATERGATE scheme for water suppression [16] while in the case of DQF-COSY [17], a low-power irradiation of the water frequency was used during the relaxation delay. NOESY spectra were recorded with mixing times of 150 ms. Processing and analyses of the spectra were done using the Bruker XWINNMR. ^1H and ^{13}C chemical shifts were referenced to the methyl resonance of 4,4-dimethyl-4-silapentane-l-sulphonic acid (DSS), used as an external standard. Assignments for $^1H/^{13}C$ resonances of aromatic side chains in lysozyme or AZB-modified lysozymes were performed using DQF-COSY, NOESY and ^1H–^{13}C HSQC spectra and checked by referring to the previously reported values [18,19].

2.8. Enzyme assay

Michaelis–Menten constant (K_m) and maximum initial rate (V_{max}) of HEWL were determined using cell wall of *Micrococcus lysodeikticus* as a substrate [20]. The reaction mixture, which consist of HEWL (0.036 g/1) and substrate (0.098–0.38 g/1) in 0.07 M phosphate buffer, pH 7.0 containing 0.017 M NaCl, was incubated at 25 °C. Time course of the lysis of cell wall was measured by recording the change in absorbance at 450 nm.

3. Results

3.1. Azobenzene-modified HEWL (AZB-L)

After the amidation of HEWL with 4-(phenylazo) benzoic acid, the main product was isolated by a combination of cation exchange and reversed phase (C18) column chromatography. The main product migrated faster on the cation exchanger column and slower on the C18 column than unreacted HEWL. As shown in Fig. 2, p*I* of the main product is lower by 0.1 than that of native HEWL. In MALDI-TOF MS analysis (Fig. 3A), the mass number of pseudomolecular ion $[M+H]^+$ of the main product is 14516 which is higher by 210 than that of the native HEWL. The mass number *m*/*z* 210 corresponds to the molecular weight of AZB residue. In the analysis of reduced triptic peptides (Fig. 3B), the peak observed at the mass number *m*/*z* 2678 is consistent with Gly22 ∼ Arg45 in HEWL. In the peptide fragment, the amino acid residue which undergoes amidation with 4-(phenylazo) benzoic acid is Lys33 only. In the main product, the mass number of the peak corresponding to the same triptic peptide is *m*/*z* 2888 which is also higher by

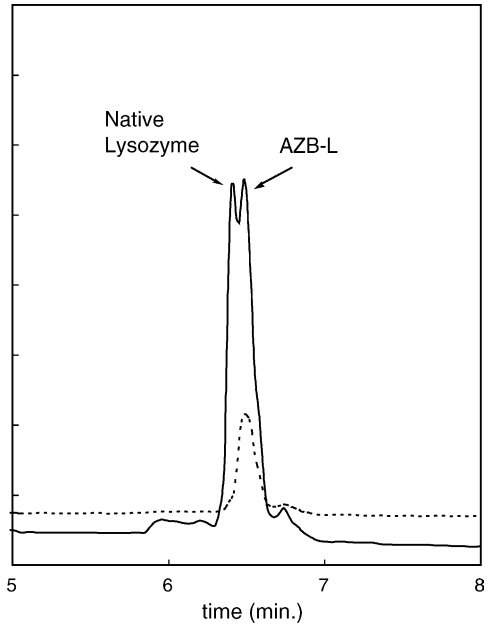

Fig. 2. Analysis of isoelectric focusing (IEF) of the native HEWL and AZB-L by capillary electrophoresis. Isoelectric point (p*I*) of the native HEWL was higher by 0.1 than that of AZB-L. Isoelectric focusing was monitored at 280 (—) and 340 (- - -) nm. The calibration of p*I* was performed using synthetic p*I* markers.

210. These results revealed that the main product is a modified HEWL (AZB-L) in which ε-amino group of Lys33 is amidated with one molecule of AZB.

3.2. Photochromism of AZB-Ls

The AZB-L exhibits reversible photochromic properties. The prepared AZB*t*-L (more than 98% in purity) was stable

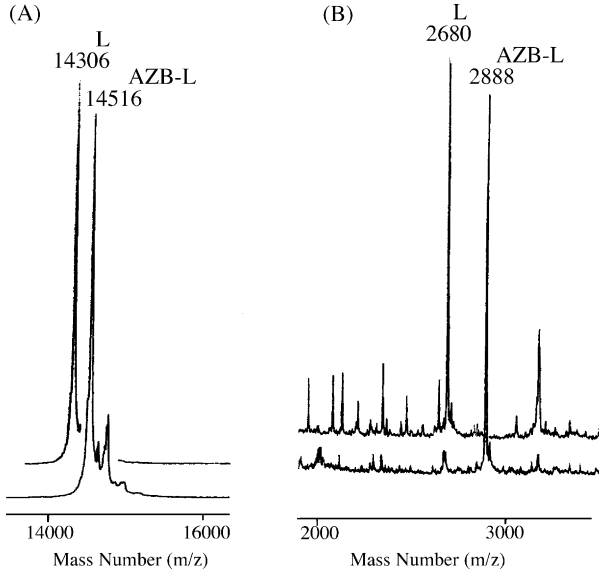

Fig. 3. Mass analyses (MALDI-TOF MS) of (A) azobenzene-modified lysozyme and (B) its tryptic peptide.

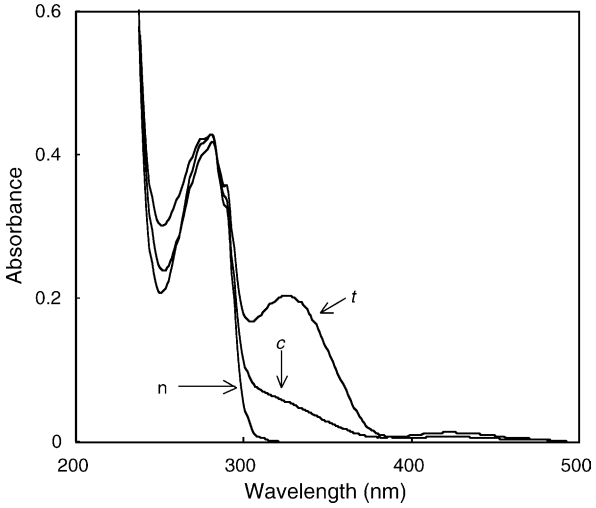

Fig. 4. Absorption spectra of (*n*) native lysozyme, (*c*) modified *cis*-azobenzen lysozyme (AZB*c*-L), and (*t*) modified *trans*-azobenzene lysozyme (AZB*t*-L). The measurements were carried out at the concentration of 11 μM in distilled water.

at room temperature in the dark in aqueous solution. On irradiation with UV light, photoisomerization from the *trans*-form to the *cis*-form (AZB*c*-L) resulted in the disappearance of absorption band at $\lambda_{max} = 320$ nm and the increase of absorption band at $\lambda_{max} = 420$ nm, corresponding to AZB*c*-L (Fig. 4). These photoisomers indicated absorption bands characteristic of AZB chromophore [12]. The composition of AZB*c*-L and AZB*t*-L was determined on a reversed phase HPLC. As shown in Fig. 5, the eluate gave three peaks. Absorption spectrum of each peak revealed that contents in the first and the second peaks were AZB*c*-Ls and that in the third peak was AZB*t*-L. The AZB*c*-Ls were tentatively named AZB*c*1-L and AZB*c*2-L in order of elution from the C18 column. At the photostationary state at 366 nm, the composition of AZB*c*1-L, AZB*c*2-L and AZB*t*-L in the *cis*-fraction was 50, 30, and 20%. Further irradiation with VIS light restored the absorption band at $\lambda_{max} = 320$ nm and depleted the band at $\lambda_{max} = 420$ nm, implying that AZB*t*-L was regenerated from AZB*c*-Ls. This result indicates that, even in lysozyme molecule, the photochromic *trans–cis* isomerization occurs. At the photostationary state at 420 nm, the reversed *trans*-fraction is comprised of 14% of AZB*c*1-L, 9% of AZB*c*2-L, and 77% of AZB*t*-L. The cycle of forward and backward reactions was repeated six times. The reproducibility of features of chromatogaram and absorption spectra indicated that the proportion of isomers at the photostationary state were reproducible.

Under the conditions in the dark and at room temperature, the thermal isomerizaton dose not occur and compositions of AZB-Ls in each fraction can be kept constant at least for 48 h.

3.3. Enzyme activity

Because it is difficult to isolate AZB*c*1-L, AZB*c*2-L, and AZB*t*-L, the measurement of enzyme activity was performed

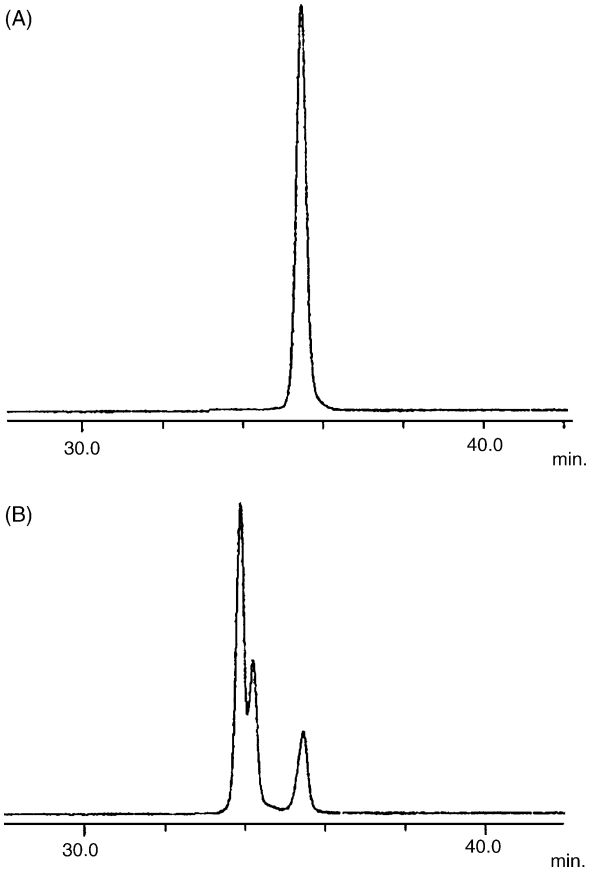

Fig. 5. Separation of azobenzene-modified lysozymes. (A) *trans*-azobenzene lysozyme (AZB*t*-L). (B) components contained in *cis*-azobenzene lysozyme (AZB*c*-L) fraction which was obtained by irradiation with UV light to AZB*t*-L of (A). The first and second peaks were generated after the irradiation and both contained components which showed the typical absorption spectrum of AZB*c*-L. Each peaks was named tentatively AZB*c*1-L and AZB*c*2-L in the order of elution from a reversed phase HPLC. The fraction was consisted of AZB*t*-L (20%), AZB*c*1-L (50%), and AZB*c*2-L (30%). Chromatographic conditions were described in the text.

using the initial AZB*t*-L and the following AZB*c*-Ls fraction at the photostationary state. The characteristics of enzyme reaction of AZB*t*-L and AZB*c*-Ls must be reflected best by the fraction which contains each component most abundantly.

The photochromism of AZB changed enzyme kinetic parameters, maximum initial rate (V_{max}), Michaelis–Menten constant (K_m), catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m), which are summarized in Table 1. By the modification with AZB, catalytic efficiency (k_{cat}/K_m) of AZB*t*-L and AZB*c*-L were lowered to one-half of the native HEWL. V_{max} and K_m values of AZB*t*-L were 1.50×10^{-1} mg ml^{-1} s^{-1}, 3.00×10^{-1} mg ml^{-1}, respectively. The fraction of AZB*c*-Ls showed 1.05×10^{-1} of V_{max} and 1.82×10^{-1} of K_m.

3.4. Conformational study

HEWL consists of 129 amino acid residues and is internally cross-linked by four disulfide bonds. The polypeptide

Table 1
Enzyme kinetic parameters of native and modified HEWLs

	V_{max} (mg ml^{-1} s^{-1})	K_m (mg ml^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mg^{-1} ml s^{-1})	R
Native	0.264	0.250	7.33	29.3	1
AZB*t*-L	0.150	0.300	4.17	13.9	0.47
AZB*c*-Ls	0.105	0.182	2.91	16.0	0.55

The *trans*-form azobenzene modified lysozyme (AZB*t*-L) was chromatographically pure. The *cis* form azobenzene modified lysozymes (AZB*c*-Ls) were a mixture of AZB*c*l-L, AZB*c*2-L, and AZB*t*-L and each compornent comprises 50, 30, and 20%, respectively. Catalytic constant of enzyme is designates as k_{cat}. R means the ratio of enzyme's catalytic efficiencies (k_{cat}/K_m) between native and modified lysozymes.

chain forms five helical segments as well as three-stranded antiparallel (β-sheet that comprises much of one wall of the binding cleft [21]. Photoinduced isomerization of AZB proceeds with large structural change of its molecule (Fig. 1). The distance between C4 and C4′ atoms in AZB decrease from 9.0 Å in the *trans*-form to 5.5 Å in the *cis*-form [11]. However, CD spectra of the native HEWL and AZB-Ls agreed well each other and any large difference in the features of ^{1}H NMR spectra was not found (Fig. 6). Both in AZB*t*-L and AZB*c*-Ls, NOE (nucler overhauser effect) between AZB molecule and protons in side chains of aromatic amino acids were not observed in ^{1}H–^{13}C 2D-NMR measurement, indicating they are locating at the distances more than 5 Å. From these results, it is clear that the modification of Lys33 by AZB does not induce the large conformational change on HEWL molecule. Amino acid residues, which changed chemical shifts by the modification with AZB and by *trans–cis* isomerization, were identified by ^{1}H 1D-NMR, focusing on indole NH proton region of Trp residues (Fig. 7 and Table 2). The clear splitting is observed in the signals of Trpl08 and Trpl23 of AZB*c*-Ls. The changes of the chemical shifts of protons occurred in AZB*c*-L were larger than those in AZB*t*-L.

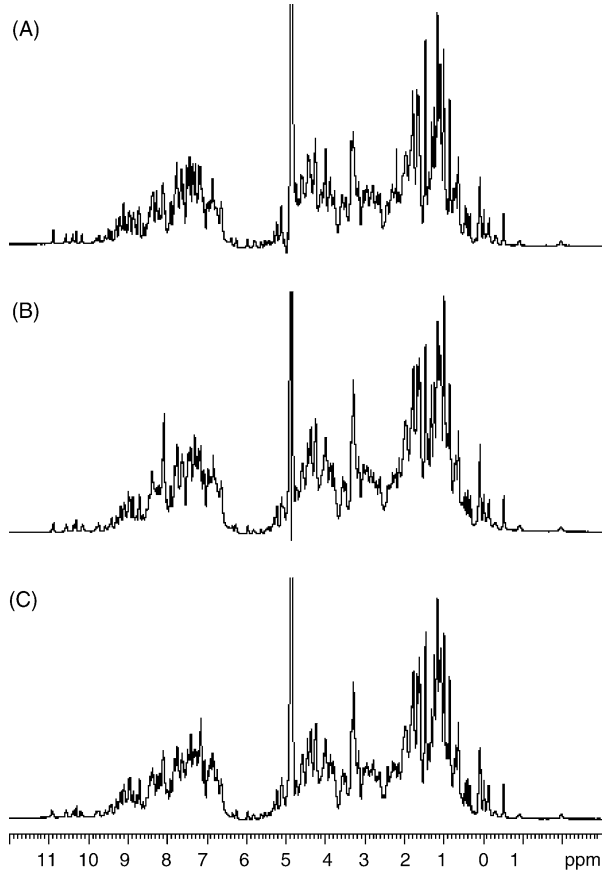

Fig. 6. Spectra of azobenzene-modified lysozymes with ^{1}H NMR spectroscopy. (A) native lysozyme; (B) modified *trans*-azobenzene lysozyme (AZB*t*-L); and (C) modified *cis*-azobenzene lysozyme (AZB*c*-L) fraction. Measurements were performed at pH 2.2 and 25 °C.

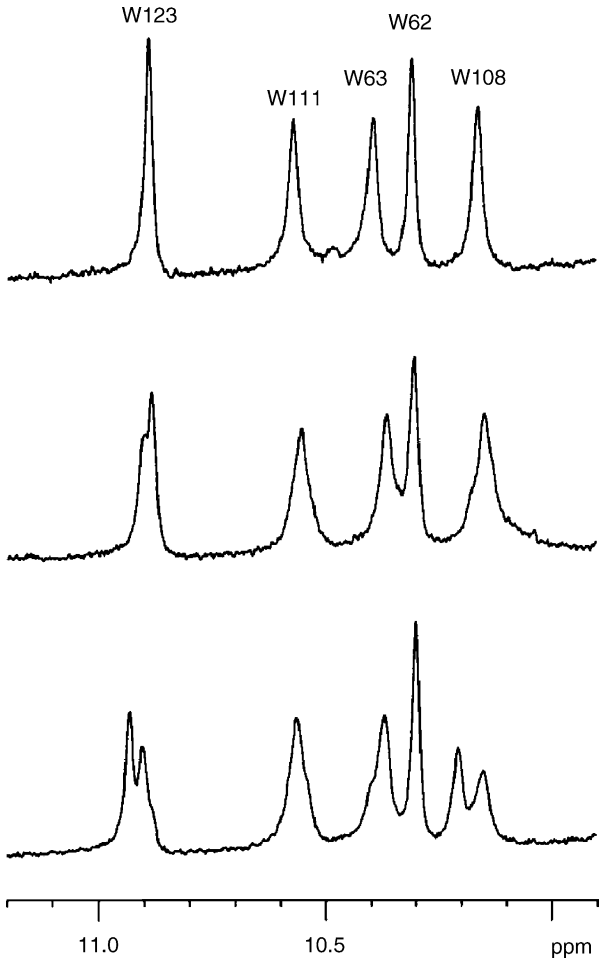

Fig. 7. Magnified ^{1}H NMR spectra at tryptophan indol NH region of azobenzene-modified lysozymes: (A) native lysozyme; (B) modified *trans*-azobenzene lysozyme (AZB*t*-L); (C) modified *cis*-azobenzene lysozyme (AZB*c*-L) fraction. Measurements were performed at pH 2.2 and 25 °C.

Table 2
Chemical shifts of tryptophan indole NH protons of lysozyme and AZB-modified lysozymes

	Lysozyme	AZB*t*-L	AZB*c*-Ls
Trp28	9.42	9.41	9.42
Trp62	10.31	10.30	10.30
Trp63	10.40	10.37	10.37
Trpl08	10.16	10.15	10.15, 10.21
Trplll	10.57	10.55	10.56
Trpl23	10.89	10.88	10.90, 10.93

Chemical shifts were in ppm referenced to DSS at 25 °C, pH 2.2.

4. Discussion

Prior to this study, we designed Leu-enkephalin analogous peptide in which Phe residue was substituted with 4-phenylazophenylalanine. However, opioid activities of the analogues carrying *cis*- or *trans*-isomers were both less than 5% of that of native Leu-enkephalin (kindly synthesized by Dr. K. Sato and assayed by Dr. Y. Ishida, Mitsubishi-Kasei Institute of Life Sciences, Tokyo). From this result, a hypothesis arises that the molecular size of AZB is too large for the opiate receptor to recognize the enkephalin analogue. In the present study, HEWL, which has a larger receptor site, was chosen and modified with AZB to study the effects of the photochromism on the enzyme activity. The modification was designed for AZB not to be introduced directly into the catalytic site and not to bring irreversible conformational change of catalytic site by discomposing the structure of enzyme.

HEWL is the most widely studied species of lysozymes and is one of the mechanistically best understood enzymes [21,22]. The enzyme destroys bacterial cell walls by hydrolyzing the β (1–4) glycosidic linkage between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) in the cell wall peptidoglycans. According to the work by D.C. Phillips et al. [22–24], in HEWL molecule, there is a binding cleft that traverses one face of the molecule. The cleft consists of six subsites (A–F) to which hexamer sugar unit of peptidoglycan is fitted. Catalytic site of the enzyme is present between D and E subsites. The extensive studies [25] revealed the "right-sided" and "left-sided" substrate binding mode as represented in Fig. 8 (Protein Data Bank accession code 1UC0). In the course of HEWL catalysis, the substrate initially binds to the left-sided binding site to form an inactive complex, next rearranges to the catalytically active right-sided one, and then is catalytically hydrolyzed. In this study, ε-amino group of Lys33 was targeted to modify with AZB, because Lys33 is a neighboring residue of a sequence of Phe34–Glu35 which are essential for the catalytic activity of HEWL [26] but the introduced AZB molecule may not protrude into the substrate binding cleft. The reacting conditions which we worked out enabled AZB-4-carboxylic acid to amidate ε-amino group of Lys33 only in HEWL. As shown in Table 1, catalytic efficiencies (k_{cat}/K_m) of AZB-Ls were lowered to one-half of that of the native HEWL. The values of V_{max} and K_m of AZB*t*-L were larger than those of AZB*c*-Ls. It is known that lower value of K_m reflects higher affinity of enzymes for the substrates. The main cause of these apparent changes of the enzyme kinetics may be the change of catalytic constant (k_{cat}). It is known that lytic activity is lowered according to the decrease of the positive charge of lysozyme molecule, because the decrease makes the binding affinity of HEWL for the negatively charged cell wall

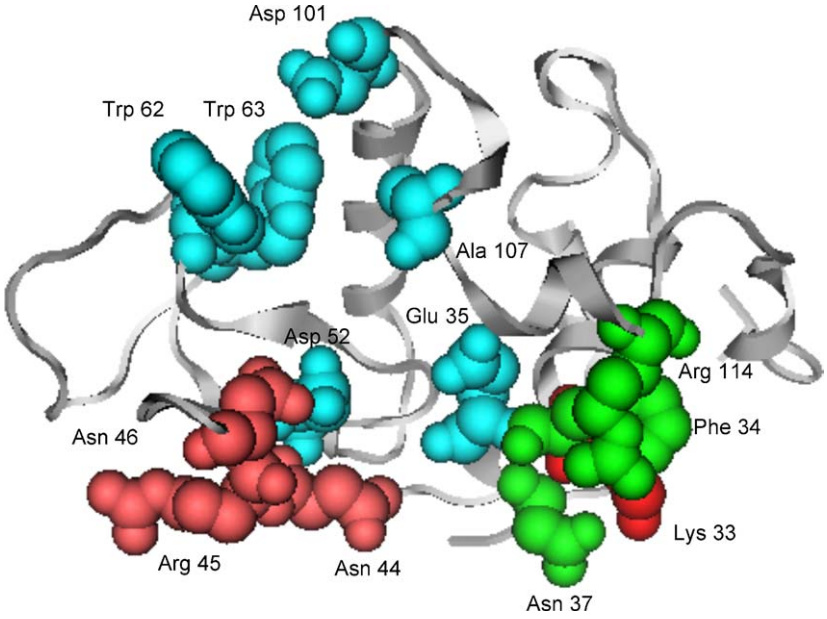

Fig. 8. A ribbon diagram of the binding site for substrate of HEWL. The main amino acid residues which constitute the site are indicated in space filling mode. Subsites from A to D (Aspl0l, Trp62, Trp63, Alal07, Asp52 and Glu35) are rendered in blue. E- and F-site saccharides initially bind to the left-sided binding site (Asn44, Arg45 and Asn46 highlighted in cherry red) to form inactive complex, next rearranged to the catalytically active right-sided one (Asn37, Phe34 and Arg114 highlighted in green). The residue highlighted in red is Lys33.

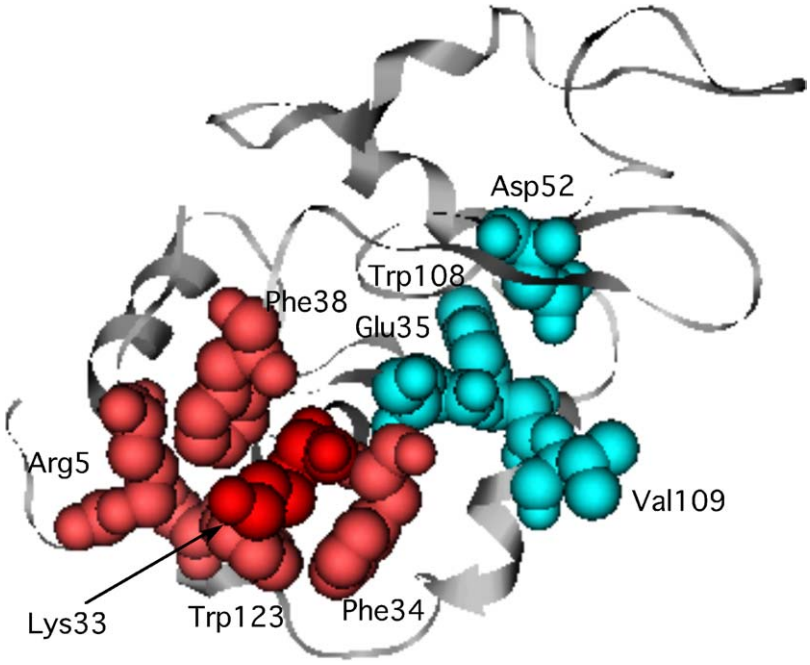

Fig. 9. A ribbon diagram of HEWL. The view is looking down around Lys33, highlighted in red. The amino acid residues (Phe38, Arg5 Trpl23 and Phe34) surrounding the side-chain of Lys33 are rendered in cherry red. The amino acid residues (Asp52, Glu35, Trpl08 and Vall09) involved in the hydrolytic reaction of lysozyme are highlighted in blue.

lower [27,28]. Indeed, by the modification of ε-amino group of Lys33 the value of p*I* of AZB-L is lower by 0.1 than that of native HEWL.

The most important feature was that the enzyme kinetics were modulated according to the photochromism of AZB. The possible mechanism of the modulation was considered, based on the results of NMR study as well as the molecular modeling of HEWL.

As shown in Fig. 9 (Protein Data Bank accession code 1UC0), lysine at residue33 is present almost in the center of the back space of F subsite, being surrounded with the side chains of Arg5, Trpl23, Phe38, and Phe34. The side chain of Lys33 is thrust toward the surface of HEWL molecule. Considering the chromatographic behaviors of AZB-Ls on a reversed phase HPLC (Fig. 5), the introduced AZB molecule, in the *trans*-form, may further stand out, resulted in the increase of the hydrophobicity of the whole lysozyme. On the other hand, by the irradiation with UV light, the photoisomerization of the AZB may proceed toward the *cis*-form as being folded inside the space. The photoisomerization may make the hydrophobicity of whole AZB*c*-L molecule lower but the local hydrophobicity of the inside of the space higher than those of AZB*t*-L. Further interesting feature was that AZB-L carrying *cis*-AZB was eluted as two peaks. This is the first finding in this field. The compositional ratio between the two peaks was reproducible during six cycles of forward and backward reactions. This suggests that the two modified lysozymes carrying *cis*-AZB coexist at equilibrium. We presume that the equilibrated two states are formed by the interaction between the attached ABZ molecule in *cis*-form and the side chains of amino acid residues surrounding Lys33 in the space.

The space locates in the right-side of HEWL [25]. Therefore, AZB*c*-L containing *cis*-isomer of AZB might not be unfavorable for the rearrangement to the catalytically active right-sided binding.

Another possible reason for the modulation is the conformational change of HEWL induced by the photochromism of AZB. But, CD and ^{1}H 1D-NMR studies showed that the modification did not bring large conformational changes in HEWL molecule (Fig. 6). The fluorescence intensities of AZB*t*-L and AZB*c*-Ls at 348 nm due to Trp residues were 20 and 40% of that of native HEWL, respectively. The decrease of fluorescence intensity indicates that the through space interaction between AZB chromophore and Trp residues are different in AZB*t*-L and AZB*c*-Ls, presumably due to the configurational changes of Trp residues. There are six residues of Trp in HEWL. It is known that the fluorescence intensity of Trp residues 62 and 108 contributes 80% of the fluorescence intensity of native HEWL [29]. In ^{1}H NMR spectrum at Trp indole NH region (Fig. 7 and Table 2), the difference of the chemical shift between AZB*t*-L and AZB*c*-Ls was observed in Trpl08 and Trpl23 but not in Trp62 and Trp63. It is notable that the clear splitting is observed in the signals of Trpl08 and Trpl23 of AZB*c*-Ls. From the results, it is assumed that the modification and the photochromism affect on the role of Trpl08 for the catalytic function of HEWL. Among the 6 Trp residues, Trp62, 63, and 108 play important roles in the catalytic action [24]. The residues, Trp62 and Trp63, are the important constituents of C subsite

to bind *N*-acetyl galactosamine through hydrogen bonds. The binding is essential to both "left-sided" and "right-sided" binding. The change of chemical shift of Trpl08 of AZB*c*-L was larger than that of AZB*t*-L. It is known that the van der Waals bond brings Trpl08 into contact with Glu35 [30]. The Glu35 transfers its proton to the O1 of the D ring distorted toward the half-chair conformation under the hydrophobic condition. The strained conformation is stabilized by a strong hydrogen bond between the D ring O6 and the backbone NH of Vall09 which is the adjacent residue of Trpl08 [30]. In this process, the hydrophobicity of Trpl08, which locates at an inner region of the right-sided binding site, plays multiple important roles, such as the maintenance of the tertiary structure, effective substrate binding, and keeping the pK_a of Glu35 abnormally high [30]. The high pK_a of Glu35 is considered to be critical for this residue to serve as general-acid catalysis in the enzyme activity of lysozyme.

From this study, it is confirmed that, by the substantial accumulation of the data obtained in this policy, photochromism is certainly expected to be more powerful tool for the analyses and the control of the function of the biological materials.

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References

[1] B.L. Feringa, W.F. Jager, B.D. Lange, Tetrahedron 49 (1993) 8267–8470.
[2] I. Willner, B. Willner, in: B.L. Feringa (Ed.), Molecular Switches, Wiley/VCH, New York, 2001, pp. 165–218.
[3] D.H. Hug, Photochem. Photobiol. Rev. 3 (1978) 1–33.
[4] K. Martinek, I.V. Berezin, Photochem. Photobiol. 29 (1979) 637–649.
[5] J. Bieth, S.M. Vartsanos, N.H. Wassermann, A.G. Cooper, B.F. Erlanger, Biochemistry 12 (1973) 3023–3027.
[6] M.A. Wainberg, B.F. Erlanger, Biochemistry 10 (1971) 3816–3818.
[7] B.F. Erlanger, N.H. Wassermann, A.G. Copper, R.J. Monk, Eur. J. Biochem. 61 (1976) 287–295.
[8] G. Montagnoli, E. Balestreri, L. Nannicini, A. Bellucci, M. Bracaloni, Int. J. Pept. Protein Res. 11 (1978) 28–32.
[9] A.D. Turner, S.V. Izzo, G.W. Rozakis, N.A. Poter, J. Am. Chem. Soc. 109 (1987) 1274–1275.
[10] M. Aizawa, K. Namba, S. Suzuki, Arch. Biochem. Biophys. 182 (1977) 305–310.
[11] I. Willner, S. Rubin, Angew. Chem. Int. Ed. Engl. 35 (1996) 367–385.
[12] I. Willner, S. Rubin, A. Riklin, J. Am. Chem. Soc. 113 (1991) 3321–3325.
[13] G.S. Kumar, D.S. Necker, Chem. Rev. 89 (1989) 1915–1920.
[14] A. Kumar, R.R. Ernst, K. Wuthrich, Biochem. Biophys. Res. Commun. 95 (1980) 1–6.
[15] G. Bodenhausen, D.J. Ruben, Chem. Phys. Lett. 69 (1980) 185–189.
[16] M. Piotto, V. Saudek, V. Sklenar, J. Biomol. NMR 2 (1992) 661–665.
[17] M. Ranee, O.W. Sorensen, G. Bodenhausen, G. Wagner, R.R. Ernst, K. Wuthrich, Biochem. Biophys. Res. Commun. 117 (1983) 479–485.
[18] Y. Wang, T.C. Bjorndahl, D.S. Wishart, J. Biomol. NMR 17 (2000) 83–84.
[19] C. Redfield, C.M. Dobson, Biochemistry 27 (1988) 122–136.
[20] D. Shugar, Biochim. Biophys. Acta 8 (1952) 302–310.
[21] T. Imoto, L.N. Johnson, A.C.T. North, D.C. Phillips, J.A. Rupley, in: P.D. Boyer (Ed.), The Enzymes, vol. 7, 3rd ed., Academic Press, New York, 1972, pp. 665–868.
[22] D.C. Phillips, Sci. Am. 215 (1966) 78–90.
[23] C.C. Blake, G.A. Mair, A.C.T. North, D.C. Phillips, V.R. Sarma, Proc. R. Soc. London, Ser. B 167 (1967) 365–377.
[24] C.C. Blake, L.N. Johnson, G.A. Mair, A.C.T. North, D.C. Phillips, V.R. Sarma, Proc. R. Soc. London, Ser. B 167 (1967) 378–388.
[25] M.R. Pincus, H.A. Scheraga, Macromolecules 12 (1979) 633–644.
[26] M. Muraki, M. Morikawa, Y. Jigami, H. Tanaka, Eur. J. Biochem. 179 (1989) 573–579.
[27] N. Yamasaki, N. Hayashi, M. Funatsu, Agric. Biol. Chem. 32 (1968) 55–63.
[28] N. Yamasaki, N. Hayashi, M. Funatsu, Agric. Biol. Chem. 32 (1968) 64–68.
[29] T. Imoto, L.S. Forster, J.A. Rupley, F. Tanaka, Proc. Natl. Acad. Sci. U.S.A. 69 (1971) 1151–1155.
[30] M. Inoue, H. Yamada, T. Yasukochi, R. Kuroki, T. Miki, T. Horiuchi, T. Imoto, Biochemistry 31 (1992) 5545–5553.