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Alteration of the substrate specificity of human lysozyme by site-specific intermolecular cross-linking

Michiro Muraki[a,*], Yoshifumi Jigami[a], Kazuaki Harata[b]

[a]*Molecular Biology Department, National Institute of Bioscience and Human-Technology, 1-1 Higashi, Tsukuba, Ibaraki 305, Japan*
[b]*Biomolecules Department, National Institute of Bioscience and Human-Technology, 1-1 Higashi, Tsukuba, Ibaraki 305, Japan*

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Abstract Human lysozyme dimers were prepared by the intermolecular cross-linking of the monomer that contained the mutation of either Arg^{41} to Cys or Ala^{73} to Cys with a divalent maleimide compound. Among the three kinds of possible dimers only R41C–R41C dimer, in which the two catalytic clefts can come close to each other due to the proximity of the conjugation site to the active sites, turned out to be 2.3 times more specific to a polymer substrate, ethylene glycol chitin, as compared to an oligomer substrate, PNP-$(GlcNAc)_5$. The result indicates that it is possible to alter the substrate specificity of an enzyme by artificially controlling the orientation of the active sites.

Key words: Human lysozyme; Intermolecular cross-linking; Site-specific; Substrate specificity

1. Introduction

Site-directed mutagenesis is a powerful means to probe the functional role of a particular residue of an enzyme [1]. On the other hand, chemical modification equip the effective ways to introduce rather large changes in chemical structure. By combining the merits of both approaches it is possible to get more detailed information on the structure–function relationships of an enzyme and to convert its catalytic properties, which are not achievable using either approach alone.

One of the most specific chemical reactions for the modification of proteins is the addition of thiol group of cysteine to an activated carbon–carbon double bond of maleimide compounds [2]. Wild type human lysozyme contains eight cysteine residues, however they form four disulfide bridges leaving no free sulfhydryl group. Therefore, if it is possible to introduce a reactive cysteine residue on the molecular surface of human lysozyme, it becomes an excellent target point for making a site-specific chemical modification including site-specific intermolecular cross-linking.

The introduction of an intermolecular cross-linkage provides the way to orient the two active clefts artificially depending on the structural position of the conjugation site. Here we report the alteration of the substrate specificity of human lysozyme by site-specific intermolecular cross-linking.

2. Materials and methods

2.1. Materials

Authentic human lysozyme was purchased from Green Cross Co., Japan and further purified using a cation exchange column (Mono S, Pharmacia) as described previously [3]. The concentration was determined on the basis of the absorption coefficient ($A^{1\%}_{280\,nm}$ = 25.65 cm^{-1}) [4]. Bis-MPHPD was from Sigma. MPB was from Boeringer Mannheim. Ethylene glycol chitin (mol.wt. = 20,000–60,000) and PNP-$(GlcNAc)_5$ were obtained from Seikagaku Kogyo, Japan. All other chemical reagents were of biochemical or analytical grade.

*Corresponding author. Fax: (81) (298) 54 6220.

Abbreviations: bis-MPHPD, *N,N'*-bis(3-maleimide propionyl)-2-hydroxy-1,3-propane diamine; MPB, *N*-(maleimide propionyl)-biocytin; PNP-$(GlcNAc)_5$, *p*-nitrophenyl-β-1,4-linked *N*-acetyl chitopentaoside.

2.2. Expression and purification of mutant human lysozyme

Mutant genes coding R41C human lysozyme and A73C human lysozyme were constructed as described previously [5]. Oligonucleotide primers for mutagenesis were, Arg^{41} to Cys, 5'-GTTACAACACTTGTGCTACTAAC-3' and Ala^{73} to Cys, 5'-GACTCCAGGCTGTGTTAACGCCTG-3'. The mismatches are shown by underlining in the primer sequence. All procedures concerning the expression and the purification of R41C human lysozyme and A73C human lysozyme were basically the same as reported previously [3]. However, 25 mM sodium phosphate (pH 7.0) containing 1 mM EDTA instead of 50 mM potassium phosphate (pH 8.0) was used as the buffer during purification in order to prevent the free sulfhydryl group of mutant enzymes from the possible air oxidation.

2.3. Estimation of free sulfhydryl group

Free sulfhydryl group content was estimated using 5,5'-dithiobis(2-nitrobenzoic acid) according to the procedure of Habeeb [6].

2.4. Intermolecular cross-linking of mutant human lysozyme

Intermolecular cross-linking of R41C human lysozyme and A73C human lysozyme is composed of a couple of steps. The first step included the reaction with excess amount of a homobifunctional maleimide compound and the isolation of the site-specifically modified human lysozyme. One milligram of either R41C human lysozyme or A73C human lysozyme (0.024 mM) was incubated at 25°C for 60 min with 0.25 mM bis-MPHPD in 25 mM sodium phosphate (pH 7.0) containing either 0.29 M (for R41C mutant) or 0.32 M (for A73C mutant) NaCl plus 1 mM EDTA. After 60 min the reaction mixture was immediately subjected to a cation exchange chromatography (Mono S, Pharmacia) and separated the components under the same gradient conditions used for the purification of mutant enzymes. The peak fraction containing the equimolar adduct of mutant human lysozyme with bis-MPHPD was pooled and stored on ice. The second step consisted of the reaction of the above equimolar adduct with R41C or A73C mutant human lysozyme and the isolation of the cross-linked dimer of human lysozyme. To the pooled fraction of bis-MPHPD modified human lysozyme described in the first step, one milligram of either R41C human lysozyme or A73C human lysozyme was added and incubated at 25°C for three hours. The reaction mixture was separated with a linear gradient of 0–0.7 M NaCl in 25 mM sodium phosphate (pH 7.0) plus 1 mM EDTA. As for the preparation of the cross-liked dimer that is composed of one R41C human lysozyme molecule and one A73C human lysozyme molecule, the equimolar adduct of R41C mutant with bis-MPHPD was further conjugated with A73C mutant.

2.5. Chemical modification of R41C mutant human lysozyme with MPB

100 μg of R41C mutant human lysozyme (0.007 mM) in 25 mM sodium phosphate (pH 7.0) plus 1 mM EDTA was incubated at 25°C with 0.2 mM MPB for 60 min. To the reaction mixture, glutathione

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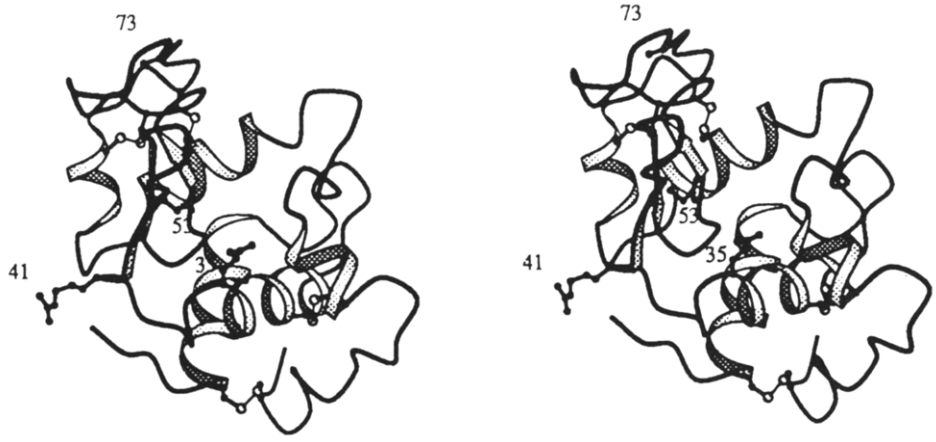

Fig. 1. Stereo view of the backbone structure of wild type human lysozyme. The side chain atoms of Arg^{41}, Ala^{73}, the catalytic residues (Glu^{35}, Asp^{53}) and all disulfide bridges are shown as a ball-and-stick model. The helix part and β-strand part are shown as a ribbon model. The picture was produced with the program MOLSCRIPT [16] using the atomic coordinates obtained from the Brookheaven Protein Data Bank (entry set 1LZ1 [17]).

(reduced form) was added (final concentration, 1 mM) and incubated for further 30 min. The R41C–MPB adduct was purified by a cation exchange chromatography (Mono S, Pharmacia) under the same gradient conditions used for the purification of other mutant human lysozymes.

2.6. Measurement of CD spectra

All measurements were performed with a JASCO J-600 spectropolarimeter at 25°C. The protein samples were dissolved in 25 mM sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl and 1 mM EDTA to give the concentration of 3.5×10^{-6} M (for dimers) or 7.0×10^{-6} M (for wild type enzyme). The measuring cell was 0.1 cm length.

2.7. Assay of enzymatic activity

Protein concentration of all mutant enzymes including the cross-linked dimers was determined from the absorbance at 280 nm using the same specific extinction coefficient, $A^{1\%}_{280\,nm} = 25.65\ cm^{-1}$, as for wild type human lysozyme. No significant difference between the protein concentration determined from the absorbance at 280 nm and that determined by the dye-binding method [7] using wild type human lysozyme as the standard was observed for the cross-linked dimers.

(1) Against ethylene glycol chitin. The activity against ethylene glycol chitin was determined basically according to the method of Hayashi et al. [8] by measuring the amount of released reducing groups. To 0.2% ethylene glycol chitin in 100 mM sodium citrate buffer (pH 5.0), was added 4 μg of lysozyme in 1/15 volume of 25 mM sodium phosphate (pH 7.0) plus 1 mM EDTA. There occurred no detectable change in pH value after the addition of lysozyme solution. The reaction mixture was kept at 37°C for 45 min and the reducing group produced was assayed colorimetrically [9].

(2) Against PNP-$(GlcNAc)_5$. 0.25 mM PNP-$(GlcNAc)_5$ was incubated with 4 μg of each sample at 37°C for 45 min in 200 μl of 100 mM sodium citrate (pH 5.0). The reaction mixture was analyzed by high-performance liquid chromatography using a Asahipak NH2-P50 column which was developed with acetonitrile/water (78:22) eluent at a flow rate of 1 ml/min, and detected at 300 nm. In the experiment to determine the ionic strength dependency of activity 10 mM sodium citrate (pH 5.0) containing the desired concentration of NaCl was used as the reaction buffer.

3. Results

3.1. Design of mutations

A couple of sort of mutations, Arg^{41} to Cys and Ala^{73} to Cys, were introduced into the molecular surface of human lysozyme. The locations of Arg^{41} and Ala^{73} in wild type human lysozyme are shown in Fig. 1. Arg^{41} is located at immediately before the short β-strand region that partly constitutes the edge of catalytic cleft. On the other hand, Ala^{73} is located in the middle of flexible loop region and is far from the active site. Both R41C mutant and A73C mutant migrated as single bands that showed the same mobilities as wild type human lysozyme in non-reducing SDS-PAGE, suggesting the native-like conformation and no direct disulfide bridge formation between the monomer molecules (Fig. 2, lanes 2–4). The free sulfhydryl groups of mutant human lysozyme were estimated using 5,5′-dithiobis(2-nitrobenzoic acid) [10]. The estimated amounts of free sulfhydryl group of R41C mutant and A73C mutant human lysozyme were 0.8 eq./mol and 0.9 eq./mol, respectively. This indicates that one cysteine residue per molecule exists as an unpaired state in either mutant, and suggests that other eight original cysteine residues form the same four disulfide bridges as wild type enzyme.

3.2. Effect of cross-linking on structure

Fig. 3 shows the separation profile of a couple of R41C human lysozyme molecules cross-linked by bis-MPHPD (R41C–R41C dimer) from the unreacted monomer by a cation

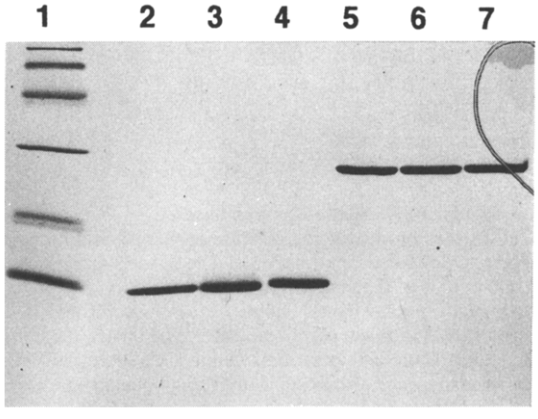

Fig. 2. SDS-PAGE analysis of mutant human lysozyme. Purified samples. 1, mol.wt. markers (14.4 kDa, 21.5 kDa, 31 kDa, 45 kDa, 66 kDa and 97 kDa); 2, wild type human lysozyme; 3, R41C mutant; 4, A73C mutant; 5, R41C–R41C dimer; 6, R41C–A73C dimer; 7, A73C-A73C dimer. The samples (2 μg each) were analyzed on SDS-PAGE (15%) under the non-reducing condition.

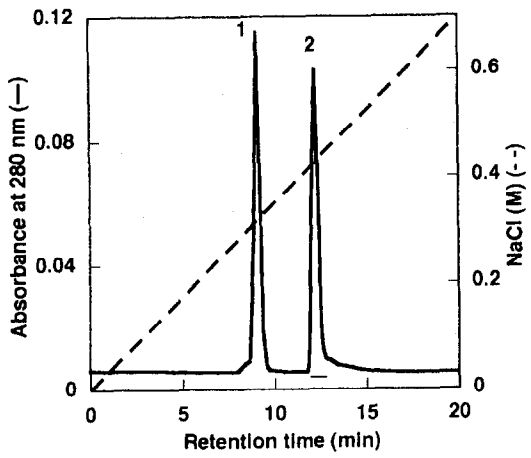

Fig. 3. Chromatographic separation of the reaction mixture for the preparation of R41C–R41C dimer. 1, monomer; 2, dimer. The dimer fraction was collected as indicated by the bar. The detailed reaction conditions and the chromatographic conditions are described in the text.

exchange chromatography. The cross-linked dimer (peak 2) eluted at a fairly higher concentration of NaCl than the monomeric human lysozyme (peak 1) due to the doubled positive charge within the whole molecule. The other dimers, a couple of A73C human lysozyme molecules cross-linked by bis-MPHPD (A73C-A73C dimer) and one R41C human lysozyme molecule and one A73C human lysozyme molecule cross-linked by bis-MPHPD (R41C–A73C dimer), were also purified to a homogeneity in the same way as R41C–R41C dimer (Fig. 2, lanes 5–7). The overall yields of cross-linked dimers were 40–45% on the basis of total amount of the used monomer.

Circular dichroism spectra of wild type and the cross-linked human lysozymes in the far-UV region were measured to monitor the effect of the cross-linking on the secondary structure (Fig. 4). The overall shape and the magnitude of spectra per monomer unit were similar among wild type monomer and all

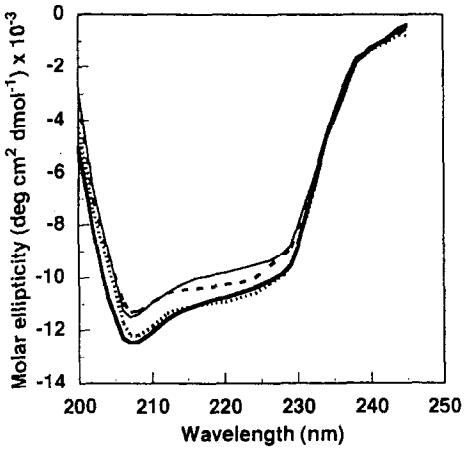

Fig. 4. Circular dichroism spectra in the far UV regions of the cross-linked human lysozyme dimers and wild type human lysozyme. ▬, R41C–R41C dimer; ····, R41C–A73C dimer; – – –, A73C-A73C dimer; —, wild type human lysozyme. Measurements were performed in 25 mM sodium phosphate (pH 7.0) containing 0.3 M NaCl and 1 mM EDTA. For the convenience of comparison the molar ellipticity of the dimers were calculated using the molar concentration of monomer unit. Protein concentration was 3.5×10^{-6} M for dimers and 7.0×10^{-6} M for wild type human lysozyme.

cross-linked dimers, indicating the integrity of the global conformation of component monomers through the cross-linking procedure.

3.3. *Effect of cross-linking on catalytic properties*

In Table 1, the relative activities of mutant human lysozymes as the percentage of that of wild type enzyme against ethylene glycol chitin and PNP-$(GlcNAc)_5$ were summarized. The optimal pH for wild type human lysozyme against these substrates [11,12] and the sufficient initial substrate concentration to saturate the active site [8,12] were used in the assays for both substrates. R41C mutant, all cross-linked dimers (per monomer unit) and R41C–MPB adduct retained almost full activity (87–95%) against ethylene glycol chitin, while the activity of A73C mutant against ethylene glycol chitin was somewhat lower (73%) as compared with other mutants. As is analogized from the result of the crystallographic study concerning hen egg-white lysozyme [13], the large flexible loop region comprising Ala^{73}, which partly constitutes the lip of the active cleft, is considered to be significantly involved in the substrate binding action of human lysozyme. Thus either the conformational change of the loop region or the inhibitory effect on the concerted motion of the flexible loop is the possible reason for the reduced activity of A73C mutant. The same is the case for the relative activities against PNP-$(GlcNAc)_5$ except that of R41C–R41C dimer. R41C–R41C dimer showed less than a half relative activity against PNP-$(GlcNAc)_5$ (38%) of that against ethylene glycol chitin (87%). As a result of this specific decrease of activity against PNP-$(GlcNAc)_5$, R41C–R41C dimer turned out to be 2.3 times more specific to a polymer substrate, ethylene glycol chitin, as compared to an oligomer substrate, PNP-$(GlcNAc)_5$ (Table 1). The ratio of the split of PNP-$(GlcNAc)_5$ to PNP-GlcNAc and $(GlcNAc)_4$ and that to PNP-$(GlcNAc)_2$ and $(GlcNAc)_3$ of R41C–R41C dimer was somewhat higher than that of either wild type or R41C mutant and the same as that of R41C–MPB adduct (Table 1). This indicates that the modification of Cys^{41} in R41C mutant with a maleimide compound slightly affected the preference in the binding mode of PNP-$(GlcNAc)_5$ to the active site, however the cross-linking itself did not affect the conformation of the active site structure. In contrast to R41C–R41C dimer, a small enhancement of the activity against oligomer substrate was observed for R41C–MPB adduct. As the phenomenon was not observed against

Table 1
Relative activity of mutant human lysozyme against ethylene glycol chitin and PNP-$(GlcNAc)_5$

Enzyme	Relative activity (%) against			
	Ethylene glycol chitin (A)	PNP-$(GlcNAc)_5$[a] (B)	(Ratio[b])	(A)/(B)
Wild type	100	100	(3.1)	1.0
R41C	90 ± 3	94 ± 2	(3.1)	1.0 ± 0.1
A73C	73 ± 3	61 ± 3	(3.3)	1.2 ± 0.1
R41C–R41C[c]	87 ± 5	38 ± 3	(3.6)	2.3 ± 0.3
R41C–A73C[c]	93 ± 5	97 ± 2	(3.0)	1.0 ± 0.1
A73C–A73C[c]	95 ± 7	90 ± 4	(3.2)	1.1 ± 0.1
R41C–MPB	89 ± 1	112 ± 4	(3.6)	0.8 ± 0.1

At least six assays were performed for each sample.
[a] Determined from the total amount of the reaction product after hydrolysis.
[b] Ratio of PNP-GlcNAc/PNP-$(GlcNAc)_2$ releasing activities.
[c] The activities of dimers are expressed as the value per monomer unit.

Table 2
Relative activity of human lysozyme dimers against PNP-$(GlcNAc)_5$ under the different ionic strength conditions

Enzyme	Relative activity[a] (%)		
	0 M NaCl	0.05 M NaCl	0.5 M NaCl
R41C–R41C	33	49	58
R41C–A73C	93	98	95
A73C-A73C	86	88	89

[a] Determined from the total amount of the reaction product after hydrolysis. The activities are expressed as the percentage of that of wild type enzyme per monomer unit.

polymer substrate, it may have resulted from the favorable interaction between the MPB moiety and the *p*-nitrophenyl group of PNP-$(GlcNAc)_5$. Table 2 shows the relative activity of cross-linked dimers against PNP-$(GlcNAc)_5$ under the different ionic strength conditions. The activity of R41C–R41C dimer was enhanced as NaCl concentration of the reaction medium increased, while that of other dimers remained essentially constant. This result suggests that the change in ionic strength of the reaction medium has a significant influence on the catalytic environment of the active sites of R41C–R41C dimer.

4. Discussion

All the cross-linked dimers showed the comparable activity (87–95%) per monomer unit to that of wild type enzyme against ethylene glycol chitin. This indicates that both catalytic sites in the cross-linked dimers are functionally active. Among the three kinds of possible dimers only R41C–R41C dimer showed less than a half relative activity (38%) of that of other dimers (97%, 90%) against an oligomer substrate (Table 1). One possible reason for this phenomenon is the difference among the preferred conformations of cross-linked dimer molecules in solution. As shown in Fig. 1, Arg^{41} is located closer to the catalytic cleft of human lysozyme than Ala^{73}. Because of this proximity of the conjugation site to the active site, the two catalytic clefts in R41C–R41C dimer can come close to each other. If the two catlytic clefts are in proximity, the liberated fragments of the substrate after the digestion in one catalytic cleft are easily accessible to the other substrate binding site. If the substrate is a polymeric substrate like ethylene glycol chitin, the liberated fragments after digestion still work as other substrates. However, in the cleavage reaction of PNP-$(GlcNAc)_5$ by human lysozyme, PNP-$(GlcNAc)_5$ is predominantly split to either PNP-GlcNAc and $(GlcNAc)_4$ or PNP-$(GlcNAc)_2$ and $(GlcNAc)_3$ [12]. The reaction product, $(GlcNAc)_4$ or $(GlcNAc)_3$, retains the similar affinity against the active site to that of $(GlcNAc)_5$ [14], but the effectiveness as a substrate differs more than 500 times between $(GlcNAc)_5$ and the shorter oligomers [15]. Under this condition the liberated fragments mainly work as competitive inhibitors of the cleavage reaction. Therefore, the proximity of the two catalytic clefts can reduce the activity against PNP-$(GlcNAc)_5$, but not that against ethylene glycol chitin. An additional evidence of the proximity of the two catalytic clefts in R41C–R41C dimer was obtained from the measurement of the relative activity against PNP-$(GlcNAc)_5$ under the different ionic strength conditions (Table 2). Here again, only R41C–R41C dimer showed the significant dependence of activity on the ionic strength of reaction medium and the activity was enhanced as the ionic strength of reaction medium increased. This phenomenon can be explained by the ionic strength dependency of the strength of electrostatic interaction. The intramolecular electrostatic interaction between the two monomer units in the cross-linked dimer becomes weaker as the ionic strength of reaction medium increases. Because of the proximity of the conjugation site to the active site in R41C–R41C dimer the change in the strength of electrostatic interaction between the two monomer units can significantly affect the spatial distance between the two catalytic clefts. The closer the two catalytic clefts locate to each other, the stronger the above mentioned inhibitory effect by the liberated fragments after digestion becomes. Accordingly, under a high ionic strength condition the two active clefts in R41C–R41C dimer are more distant from each other than under a low ionic strength condition due to the weakness of the electrostatic interaction between the two monomer units, which will result in the increase of the activity against PNP-$(GlcNAc)_5$. In the catalysis performed by the endoglycosidase such as human lysozyme either oligomer substrate or polymer substrate is hydrolyzed using the active cleft composed of the several subsites, in which each subsite recognizes the same monomeric sugar residue that is the common component of both substrates. Therefore it is generally difficult to accomplish the specific reduction of the catalytic efficiency against an oligomer substrate without damaging the activity against a polymer substrate by the direct modification of the active site. Our result indicates that the introduction of site-specific intermolecular cross-linkage provides a novel way to enhance the substrate specificity of an endoglycosidase against a polymer substrate without the significant reduction of catalytic activity.

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