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A Protein's Conformational Stability Is an Immunologically Dominant Factor: Evidence That Free-Energy Barriers for Protein Unfolding Limit the Immunogenicity of Foreign Proteins

Takatoshi Ohkuri,^{*,†} Satoko Nagatomo,^{*} Kenji Oda,^{*} Takanori So,^{*,‡} Taiji Imoto,[§] and Tadashi Ueda^{*}

Foreign protein Ags are incorporated into APCs and then degraded by endosomal proteases. The peptides are then mounted on MHC II molecules on the surfaces of APCs. The T cell-triggering response and, therefore, the immune response, were suggested to be governed by the degree of conformational stability of the foreign protein Ags. However, there is little evidence that a protein's conformational stability is an immunologically dominant factor. In this study, we show that a protein has a threshold of conformational stability to prevent the immunogenicity of foreign proteins. Inverse and linear correlations were found between the amount of IgG production against lysozymes and the free-energy change for the unfolding of lysozymes, based on the correlation between the free-energy changes of the protein unfolding and the amount of IgG production against lysozymes with different stabilities in mice using hen egg white lysozyme derivatives and mutant mouse lysozymes, in which the sequence between 107 and 116 is replaced with that of hen egg white lysozyme, which can produce autoantibodies in mice. Interestingly, the thresholds of free-energy changes for both lysozymes to prevent their immunogenicity were almost identical (21–23 kcal/mol). To confirm the results, we also showed that the cross-linking of Phl p 7, in which intact Phl p 7 has stability greater than ~20 kcal/mol under physiological conditions, induced minimal IgG production in mice, whereas intact Phl p 7 was antigenic. From the above results, we suggest that protein conformational stability was an immunologically dominant factor. *The Journal of Immunology*, 2010, 185: 000–000.

A protein usually exists in equilibrium between folded and unfolded states. Protein conformational stability is defined by the ratio of the folded state to the unfolded state. Under physiological conditions, the equilibrium greatly favors the folded state. However, proteins are inherently unstable, and the rate of transition from the folded to the unfolded state is relatively rapid. Moreover, protein molecules also undergo relatively large fluctuations in internal energy, which are reflected by conformational fluctuations (1–3). The use of therapeutic proteins, such as erythropoietin and tissue plasminogen activator, has recently revolutionized the treatment of many diseases, and many other proteins are expected to become available for therapeutic use in the future (4, 5). However, the use of therapeutic proteins is associated with the risk

for Ab formation (5–7). Protein degradation by proteases occurs via the unfolded state of a protein (8–11), and an understanding of the relationship between protein conformational stability and immunogenicity is important for the development of biopharmaceutical therapeutics (12).

The mammalian immune system has evolved so that Ag-specific CD4⁺ T cells recognize Ag-derived peptides. Protein Ags are incorporated into APCs and then are degraded by endosomal proteases (13–15). The peptides are then mounted on MHC class II molecules on the surfaces of APCs (16, 17). Because protein unfolding may be a crucial step in intracellular Ag processing, the conformational stability of the protein Ag is important in determining the immune response. In a previous study using modified hen egg white lysozyme (HEL), we found that the T cell-triggering response and, therefore, the immune response, were governed by the degree of conformational stability of the protein Ags (18, 19). This finding was supported by a study using derivatives of snake toxin with different stabilities, which found that Ag presentation was controlled by the stability of the toxin (20). When we carry out any mutations in recombinant human protein to improve protein conformational stability for long-term use or to improve protein function, there is a possibility that the mutation will induce an undesired immune response in the human body during therapeutic use. We should focus on the criteria of how much we stabilize protein conformation so that the mutated protein may not induce the undesired immune response under physiological conditions. However, few reports indicate the criteria for conformational stability of a protein to avoid immunogenicity.

In this study, we examined the correlation between free-energy changes of protein unfolding and the amount of IgG production against proteins in BALB/c mice using two homologous proteins and found the threshold of free-energy changes for the protein

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Abbreviations used in this paper: CD, circular dichroism; DSC, differential scanning calorimetry; HEL, hen egg white lysozyme; 1–15 CL-HEL, modified hen egg white lysozyme, in which K1 and H15 are cross-linked with an alkyl chain and W108 is changed to oxindolealanine108; 35–108 CL-HEL, modified hen egg white lysozyme in which E35 and W108 are cross-linked with an ester bond; double CL-HEL, modified hen egg white lysozyme in which K1 and H15 are cross-linked with an alkyl chain and E35 and W108 are cross-linked with an ester bond; ML, mouse lysozyme; 3mut-ML, mutant mouse lysozyme in which the sequence between 107 and 116 is replaced with that of hen egg white lysozyme; Tm, melting temperature.

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unfolding to prevent the immunogenicity of foreign proteins. Based on the results, we prepared a hyperstabilized grass pollen allergen, Phl p 7, and demonstrated that hyperstabilization of Phl p 7 produced minimal IgG against a foreign protein in mice. In this study, we demonstrate that the conformational stability of a protein is an immunologically dominant factor.

Materials and Methods

Preparation of HEL derivatives

HEL, purified by repeated recrystallization five times, was kindly donated by Kewpie (Tokyo, Japan). Modified HEL, in which E35 and W108 are cross-linked with an ester bond (35–108 CL-HEL) and modified HEL, in which K1 and H15 are cross-linked with an alkyl chain, and E35 and W108 are cross-linked with an ester bond (double CL-HEL) were prepared using the methods described by Imoto et al. (21) and Ueda et al. (22), respectively. Modified hen egg white lysozyme, in which K1 and H15 are cross-linked with an alkyl chain, and W108 is changed to oxindolealanine108 (1–15 CL-HEL) was prepared by hydrolysis of double CL-HEL (22).

Preparation of mouse lysozyme mutants

Site-directed mutagenesis of mouse lysozyme (ML) was performed by PCR, according to previously described methods (23). Mutations were confirmed by DNA sequence analysis. The ML mutants were expressed in *Pichia pastoris* GS115 cells harboring the recombinant plasmid constructed using a *pPIC9* vector (Invitrogen, Carlsbad, CA), as described previously (23). Purification of ML mutants was carried out according to the previously described method (23).

Preparation of Phl p 7

The Phl p 7 gene was obtained by PCR using long primers and was inserted between the NdeI and EcoRI sites of the *pET22b* vector (Merck, Darmstadt, Germany), just downstream of the T7 promoter. Site-directed mutagenesis of the Phl p 7 gene was performed by PCR, according to previously described methods (23). Mutations were confirmed by DNA sequence analysis. Phl p 7 was overexpressed in *Escherichia coli* BL21 cells harboring a recombinant plasmid constructed using a *pET22b* expression vector (Merck), as described previously (24). The cells were suspended in 25 mM MOPS buffer (pH 7), 0.2 M KCl, and 0.2 mM PMSF, and a lysate was obtained as described previously (25), with the exception that 20 mM spermidine was present during lysozyme treatment at 4°C. Ammonium sulfate (55% w/v) was added to the cell lysate for salting out. The precipitate was resuspended in imidazole buffer (25 mM imidazole [pH 7.4], 1 mM CaCl₂, 1 mM 2-ME) and passed through a cation-exchange column (1 × 50 cm; SP-Toyopearl, Tosoh, Tokyo, Japan) equilibrated with imidazole buffer. The protein fractions were applied to an anion-exchange column (1 × 50 cm; DEAE-Toyopearl, Tosoh, Tokyo, Japan) equilibrated with imidazole buffer, and the proteins were eluted with a linear gradient in the same buffer with 0.5 M NaCl. The eluted protein fractions were applied to a gel-filtration column (1 × 120 cm, Sephadex G-75; GE Healthcare UK, Buckinghamshire, U.K.) equilibrated with imidazole buffer. Phl p 7 was judged to be pure by SDS-PAGE.

Phl p 7 forms dimers and possesses a free cysteine residue at position 64 of each subunit. M5 in the N-terminal region is located near C64' in another subunit. To create an intersubunit disulfide bond between M5 and C64' (cross-linking dimer), M5C mutant Phl p 7, in which Met5 of Phl p 7 is mutated to Cys, was prepared from *E. coli*. The M5C mutant Phl p 7 was purified using imidazole buffer in the absence of 2-ME. The formation of an intersubunit disulfide bond in the M5C mutant Phl p 7 was assessed by reducing and nonreducing SDS-PAGE. The M5C mutant Phl p 7 under nonreducing conditions was observed as a single band on SDS-PAGE with an apparent size of 17 kDa, consistent with the size of a dimer of Phl p 7 (8.6 kDa). Moreover, M5C mutant Phl p 7 was observed as two peaks on reversed-phase HPLC after treatment with cyanogen bromide. The two peaks were identified as peptide fragment 2–43 + 45–71 (digested at C-terminal of M1, M43, and M44) and peptide fragment 72–78 (digested at C-terminal of M71), using an amino acid analyzer (Hitachi L-8500, Hitachi, Tokyo, Japan). This confirmed the formation of an intersubunit disulfide bond in the M5C mutant Phl p 7.

Differential scanning calorimetry measurements

Differential scanning calorimetry (DSC) measurements and data analyses were carried out using a VP-DSC (GE Healthcare UK) system equipped with a Gateway personal computer, as described in the literature (26). The scan rate was 1.0°C/min. The sample solutions were prepared by dissolution in 0.05 M glycine buffer (pH 3), and the protein concentration was 20 µM. Data analysis was performed using Origin software (GE Healthcare UK).

Measurement of serum Ab levels

Six-week-old female BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan). The immunization experiments using lysozyme were performed according to the methods of So et al. (19), with slight modification. Five mice were injected i.p. with 0.1 ml saline emulsified 1:1 in CFA containing HEL derivatives or mutant mouse lysozyme in which the sequence between 107 and 116 is replaced with that of hen egg white lysozyme (3mut-ML; 50 µg/mouse) on day 0. They received s.c. booster immunizations with the same protein Ag in IFA on day 35. Blood samples were drawn from the orbital sinus into capillary tubes every week after immunization. The immunization experiments using Phl p 7 were performed according to the methods of Niggemann et al. (27), with slight modification. Five mice were injected i.p. with 50 µg wild-type Phl p 7 or M5C mutant Phl p 7 (50 µg/mouse) adsorbed to 2 mg aluminum hydroxide on days 0, 7, 14, 21, 28, 35, and 42, and blood samples were drawn from the orbital sinus into capillary tubes every week. Serum IgG levels to each protein Ag were determined by ELISA, according to previously described methods (19).

Measurement of T cell proliferation

Mice were immunized s.c. with wild-type Phl p 7 in 0.1 ml CFA. These mice were euthanized 9 d after immunization; draining lymph node cells were removed, and cells were suspended in serum-free HL-1 medium (Bio-Whittaker, Walkersville, MD) supplemented with L-glutamine (216 µg/ml), 2-ME (5 × 10⁻⁵ M), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were cultured in a flat-bottom 96-well plate (2 × 10⁵ cells/100 µl) and 20 µl Ag solution (19.4, 2.15, 0.24, 0.026, or 0.003 µM) was added. After 4 d of incubation at 37°C, the number of viable cells in each cell was determined by a WST-1 cell-proliferation assay system (TAKARA, Tokyo, Japan), according to the manufacturer's instructions, and by measuring the absorbance at 490 nm.

Degradation assay of Phl p 7

Degradation of Phl p 7 was measured using the rabbit reticulocyte lysate assay (28). Wild-type Phl p 7 or M5C mutant Phl p 7 at a protein concentration of 2.5 mg/ml in 2 µl aqueous solution was incubated at 37°C with 10 µl rabbit reticulocyte lysate (Promega, Madison, WI) for 0, 3, 5, or 7 d. Proteins were detected by Western immunoblotting and quantified using Scion imaging software (National Institutes of Health, Bethesda, MD).

Results

Preparation of modified lysozymes and their stabilities

It has been reported that HEL was stabilized by cross-linking between K1 and H15 with an alkyl chain (1–15 CL-HEL) (29) and by cross-linking between E35 and W108 through an ester bond (35–108 CL-HEL) (21, 30). These modified amino acids are shown in the three-dimensional structure of HEL (Fig. 1A). Moreover, intramolecular double cross-links of 1–15 CL and 35–108 CL greatly increased the stability of HEL (double CL-HEL) (22). We prepared three HEL derivatives with different stabilities using previously described methods. The thermal stabilities of the HEL derivatives were analyzed using DSC at acidic pH, where the denaturation of the wild-type HEL is reversible (Fig. 2A). The thermodynamic parameters for denaturation of lysozymes are shown in Table I. Three HEL derivatives were more stable than the wild-type HEL, and double CL-HEL was particularly stable. These results were similar to our previous results (22).

It was reported that autoantibodies against ML were strongly induced in BALB/c mice immunized with an ML mutant substituted at Ala114, His115, and Gln117 with Asn, Arg, and Lys, respectively (3mut-ML), in which the core immunodominant T cell epitope of HEL 107–116 was introduced into ML (31). Using random mutagenesis, we previously obtained Y63H ML, which demonstrated increased thermal stability compared with wild-type ML (T. Ueda, N. Ohtsuka, Y. Hashimoto, and T. Imoto, unpublished observations). Furthermore, Y63R or Y63A mutation was introduced into 3mut-ML (Y63R 3mut-ML or Y63A 3mut-ML) to alter its conformational stability. These mutated amino acid residues were plotted on a three-dimensional structure of ML (Fig. 1B). The

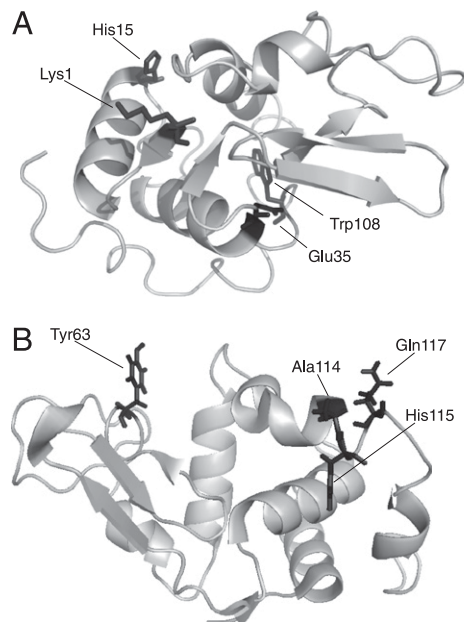


FIGURE 1. A, Three-dimensional structure of wild-type HEL (from Brookhaven Protein Data Bank entry, 1LZA). The chemically modified amino acids are shown as sticks. B, Three-dimensional structure of wild-type ML (from Brookhaven Protein Data Bank entry, 1IVM). The mutated amino acids are shown as sticks.

stabilities of these mutants were measured using DSC under the same conditions as the HEL derivatives (Fig. 2B). As shown in Table I, the melting point (T_m) of Y63H 3mut-ML was $\sim 5^\circ\text{C}$ higher than that of 3mut-ML, the T_m of Y63R 3mut-ML was $\sim 3^\circ\text{C}$ higher than that of 3mut-ML, and that of Y63A 3mut-ML was $\sim 4^\circ\text{C}$ lower than that of 3mut-ML. The alteration of the conformational stability of the 3mut-ML mutants may be due to changes in their hydrophobicity and electrostatic interactions. Thus, three 3mut-ML mutants possessing different conformational stabilities were constructed.

Immunogenicity of modified lysozymes

To determine the effect of protein conformational stability on IgG production, BALB/c mice were immunized with the HEL and 3mut-ML mutants emulsified in CFA and were bled every week. We used the Freund's adjuvant to evaluate the difference in IgG response between immunizing proteins, according to a previous study (19). The reactivities of these antisera to the respective HEL derivatives or 3mut-ML mutants were analyzed using ELISA. The IgG response order to the HEL derivatives after 42 d was wild-type HEL > 1–15 CL-HEL > 35–108 CL-HEL > double CL-HEL (Fig. 3A). When IgG production of 1–15 CL-HEL and that of 35–108 CL-HEL were reported previously, the order of T cell-stimulating capacity was wild-type HEL > 1–15 CL-HEL > 35–108 HEL (18). Thus, the IgG production results were consistent with the T cell-stimulating capacities of the HEL derivatives. In this study, highly stable double CL-HEL was also used to examine the correlation between protein conformational stability and IgG production in more detail. The results demonstrated that greater increases in conformational stability of protein Ag led to further reductions in the immune response (Fig. 3A). Moreover, the amount of anti-HEL IgG was inversely and linearly correlated with the free-energy change in thermal denaturation for each protein (filled squares in Fig. 4). A similar result was also obtained in the experiment using the 3mut-ML mutant. The IgG response order to the ML mutants after 42 d was Y63H 3mut-ML < Y63R 3mut-ML < 3mut-ML < Y63A 3mut-ML (Fig. 3B), and the plots of the amount

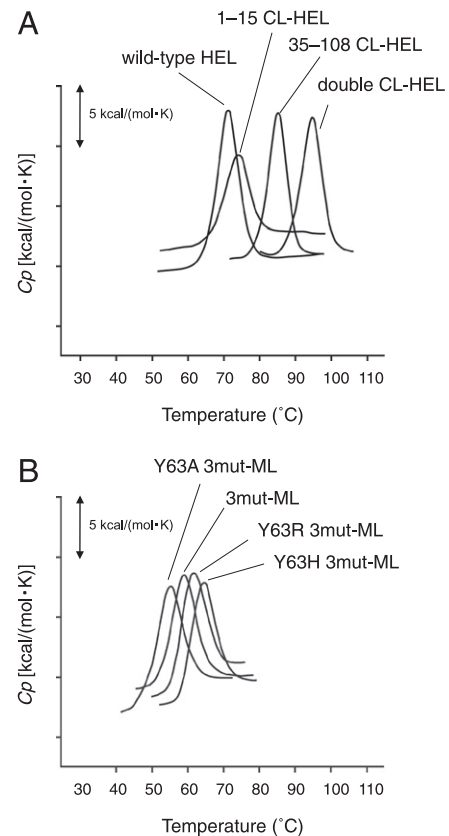


FIGURE 2. Thermal denaturation of HEL derivatives and ML mutants. A, DSC profiles of the wild-type HEL, 1–15 CL-HEL, 35–108 CL-HEL, and double CL-HEL. B, DSC profiles of 3mut-ML, Y63A mut-ML, Y63R mut-ML, and Y63H mut-ML. Protein samples were 20 μM in 0.05 M glycine buffer (pH 3). The scan rate was 1.0 K/min. DSC measurements were carried out using a MicroCal VP-DSC system, and data analysis was performed using Origin software.

of IgG production are inversely and linearly correlated with protein stability, similar to the case with HEL derivatives ($\circ$ in Fig. 4). Interestingly, when a linear extrapolation in the plots yields the value of free-energy change for the thermal denaturation in which IgG production is zero, the value for 3mut-ML mutants (23.1 kcal/mol) was very close to that of the HEL derivative (21.1 kcal/mol). Despite the fact that the values for free-energy change of the HEL derivatives and 3mut-ML mutants were obtained under acidic conditions, they correlated with the amount of IgG production observed under physiological conditions. This correlation is not unreasonable, because proteins are degraded within intracellular acidic organelles by proteases, such as cathepsins B and D, prior to Ag-specific T cell activation (32, 33). The fact that two independent experiments demonstrated a good correlation between an increase in protein conformational stability and a decrease in IgG production suggests that hyperstabilization of a protein Ag can lead to avoidance of the immune response against Ag. Proteins generally have a stability of 5–20 kcal/mol under physiological conditions. Therefore, if a protein with a stability >20 kcal/mol could be prepared, it might be able to prevent the immune response. To confirm this idea, we tried to prepare a hyperstabilized protein, as follows.

Decrease in Phl p 7 immunogenicity by hyperstabilization

The grass pollen allergen Phl p 7 consists of two calcium-binding domains (EF-hands). Its three-dimensional structure consists of two monomers assembled in a head-to-tail arrangement (Fig. 5) (34), and the structure of the protein is hyperthermostable (35). We

Table I. Thermodynamic parameters for denaturation of lysozymes at pH 3

Lysozyme	Tm ^a (°C)	ΔTm (°C)	ΔG ^b (kcal/mol)	ΔH ^b (kcal/mol)	ΔS ^b (kcal/mol/K)
Wild-type HEL	69.5	±0	17.1	74.6	0.21
1–15 CL-HEL	74.6	+5.1	17.5	56.0	0.14
35–108 CL HEL	85.4	+15.9	18.9	75.9	0.21
Double CL-HEL	94.0	+24.5	21.8	63.4	0.15
3mut-ML	61.7	±0	15.5	77.7	0.23
Y63A 3mut-ML	58.0	−3.70	13.9	74.3	0.22
Y63R 3mut-ML	65.1	+3.36	17.6	83.6	0.24
Y63H 3mut-ML	66.8	+5.13	18.8	87.0	0.25

SD for ΔG, ΔH, and ΔS of the wild-type HEL at 37°C are 1.0, 2.1, and 0.12, respectively.

^aTemperature in which the half part of protein is denatured.

^bThermodynamic parameters for the denaturation at 37°C.

determined whether a decrease in IgG production could occur through increased stabilization of the protein Ag, even in an already stable protein like Phl p 7. Intersubunit disulfide engineering has been successful in enhancing the stability of some dimeric proteins (36–39). Phl p 7 has one cysteine residue, located at position 64 on the subunit interface. The crystal structure analysis showed that Met 5' is located near Cys 64, with a distance between the β-carbons of these amino acids ~5 Å (Fig. 5). We attempted to introduce a disulfide bond between Met5' when it was replaced with a cysteine residue and the inherent free-cysteine residue of Cys64 to create a hyperstabilized Phl p 7 mutant. The M5C mutant Phl p 7 was then prepared from *E. coli*. The formation of an intersubunit disulfide bond in purified M5C mutant Phl p 7 was confirmed by reducing and nonreducing SDS-PAGE and by peptide analyses of a cyanogen bromide digest of Phl p 7 by reversed-phase HPLC (described in *Materials and Methods*). The stability of the M5C mutant Phl p 7 was evaluated by guanidine-induced equilibrium unfolding as a change in ellipticity at 220 nm. Although the wild-type Phl p 7 was partially unfolded in 6 M guanidine hydrochloride at pH 7, the M5C mutant Phl p 7 was hardly denatured, even at high guanidine hydrochloride concentrations (Fig. 6A). This indicated that the M5C mutant Phl p 7 was hyperstabilized as a result of the introduction of an intersubunit disulfide bond. The enhanced stability of Phl p 7 due to the M5C mutant was also confirmed under acidic conditions at pH 5 (data not shown). We were unable to evaluate the free-energy change for denaturation at neutral or mildly acidic conditions, because denaturation was incomplete even at high guanidine concentrations. To evaluate the free-energy change for denaturation, the guanidine-induced equilibrium unfolding of the M5C mutant Phl p 7 was conducted at pH 10, an

extreme condition for this protein. The unfolding transition of the M5C mutant Phl p 7 at pH 10 was monophasic and highly cooperative (Fig. 6B). In the guanidine-induced equilibrium unfolding at pH 10, guanidine HCl concentration in which the half part of protein is denatured of the M5C mutant Phl p 7 and that of the wild-type Phl p 7 were 5.3 and 2.2 M, respectively, and the free-energy changes for the unfolding were 19.4 and 12.6 kcal/mol, respectively. These results suggest that the stability of Phl p 7 was remarkably enhanced by cross-linkage in the M5C mutation. Based on these results, the M5C mutant Phl p 7 demonstrated unfolding stability > 20 kcal/mol at pH 7 or 5.

To examine whether a hyperstable protein, M5C mutant Phl p 7, was associated with a decrease in IgG production, BALB/c mice were immunized with M5C mutant Phl p 7 and wild-type Phl p 7 in aluminum adjuvant and were bled every week, according to previously described methods with slight modifications (27). The reactivities of these antisera to M5C mutant Phl p 7 and wild-type Phl p 7 were analyzed using ELISA. Serum IgG levels to each protein Ag are shown in Fig. 7A. Although wild-type Phl p 7 stimulated IgG production, the M5C mutant Phl p 7 stimulated minimal IgG production. We also examined whether the M5C mutation was involved in T cell epitopes of Phl p 7; the T cell epitopes of Phl p 7 in BALB/c mice (H-2^d haplotype) have not been reported. Neither wild-type peptide 1–20 nor M5C mutant peptide 1–20 of Phl p 7 activated T cells, whereas the whole Phl p 7 activated T cells under the same conditions (Fig. 7B). Moreover, we confirmed that the conformational B cell epitopes of Phl p 7 are unaffected by the formation of an intersubunit disulfide bond (Supplemental Fig. 1). From analysis of secondary structure of the wild-type and M5C mutant Phl p 7 using circular dichroism, we found that the M5C mutant Phl p 7 had the same secondary structure as the wild-type Phl p 7, suggesting that the S–S bond in the M5C mutant Phl p 7 has minimal effect on the structural rearrangements of intact Phl p 7 (Supplemental Fig. 2). These results, together with the correlation between protein conformational stability and IgG

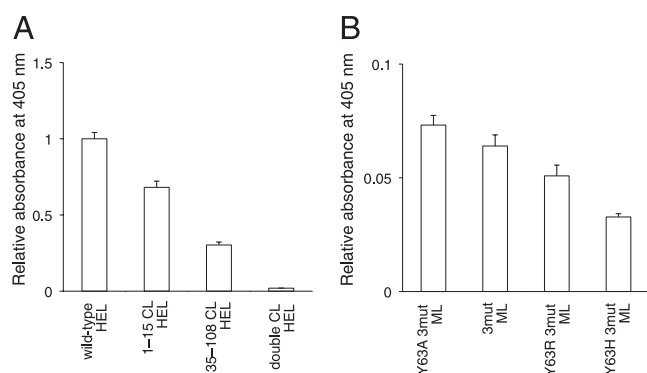


FIGURE 3. Measurement of serum IgG levels by ELISA. Eight groups of five mice each were immunized with wild-type HEL, 1–15 CL HEL, 35–108 CL HEL, or double CL HEL (A) and 3mut-ML, Y63A 3mut-ML, Y63R 3mut-ML, or Y63H 3mut-ML (B). The data show IgG levels in the serum after day 42 and are expressed relative to the value of absorbance at 405 nm in wild-type HEL.

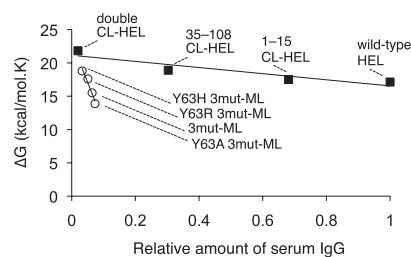


FIGURE 4. Correlation between protein conformational stability and Ab production by HEL derivatives (○) or 3mut-ML mutants (●). The data correspond to the free-energy change for thermal denaturation in Table I and serum IgG levels in Fig. 3.

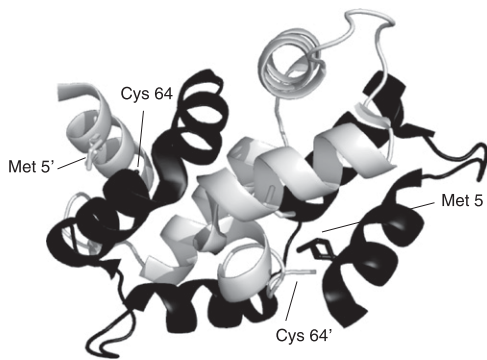


FIGURE 5. Dimeric structure of wild-type Phl p 7 (from Brookhaven Protein Data Bank entry, 1K9U). Each subunit is shown in black or white. The mutated amino acids are shown as sticks, and the prime symbol indicates the residue from the other subunit.

production described above, led us to conclude that hyperstabilization of a protein depressed the immune response.

Proteolytic-degradation analysis *in vitro* previously showed that restriction of denaturation by increasing the stability of lysozyme prevented the generation of antigenic peptides, resulting in the suppression of T cell activation (18). Generally, protein Ags are degraded in the cell by proteases, such as cathepsins B and D, prior to Ag-specific T cell activation (32, 33). Although we examined the resistance of M5C mutant Phl p 7 and wild-type Phl p 7 to cathepsin B or D digestion, Phl p 7 was not readily cleaved because of its intrinsic stability (data not shown). To confirm the relationship between stability and protein degradation, M5C mutant Phl p 7 and wild-type Phl p 7 were subjected to *in vitro* degradation assays using rabbit reticulocyte lysate (28). Each residual protein was detected by Western immunoblotting and was quantified using Scion imaging software (Fig. 8A). As shown in Fig. 8B, we confirmed that the image analysis was quantitative. M5C mutant Phl p 7 was stable for 120 h within rabbit reticulocytes, whereas wild-type Phl p 7 was degraded by 67, 46, and 43% at 24, 72, and 120 h of incubation, respectively. Moreover, we examined the proteolytic degradation of Phl p 7 with pronase, according to the literature (40). Phl p 7 and

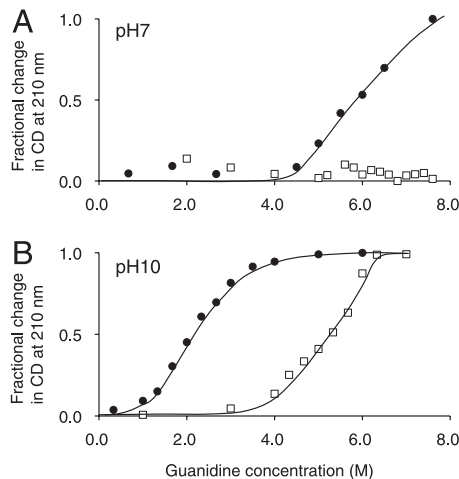


FIGURE 6. Fractional change in CD at 220 nm for the wild-type Phl p 7 (●) or M5C mutant Phl p 7 (□) in 20 mM phosphate buffer containing 1mM CaCl_2 at pH 7 (A) and in 50 mM NaHCO_3 -NaOH buffer containing 1mM CaCl_2 at pH 10 (B) in the presence of various concentrations of guanidine hydrochloride. CD spectra were obtained with a Jasco-J 720 spectropolarimeter. These experiments were carried out at 37°C. The concentration of each protein was 10 μM . CD, circular dichroism.

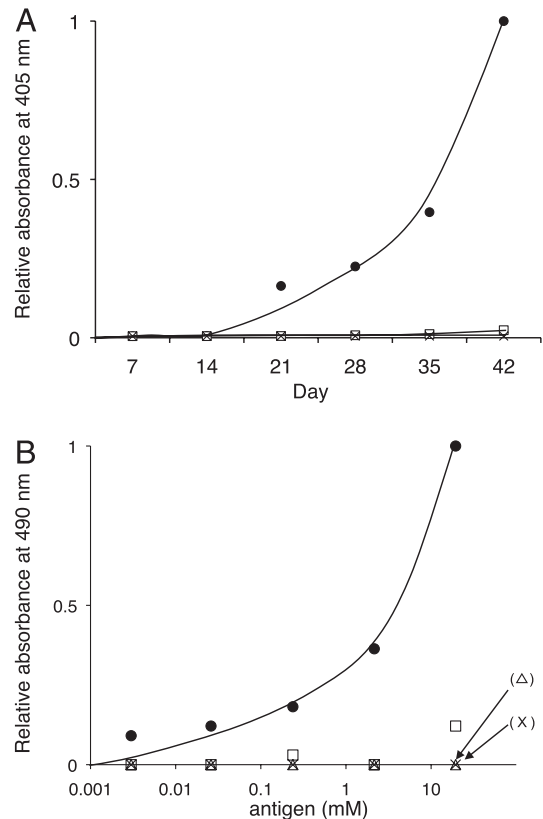


FIGURE 7. A, Detection of anti-Phl p 7 Abs in BALB/c mice immunized with wild-type Phl p 7 (●), M5C mutant Phl p 7 (□), and PBS as control (X). B, Lymph node T cell-proliferation assay. BALB/c mice immunized with wild-type Phl p 7 (●), M5C mutant Phl p 7 (□), wild-type peptides 1–20 (X), and M5C mutant peptides 1–20 (Δ).

M5C mutant Phl p 7, at a protein concentration of 0.2 mg/ml in 0.1 M NH_4HCO_3 buffer (pH 8), were incubated at 37°C with pronase at a protein concentration of 0.04 mg/ml for 4 h. The quantity of

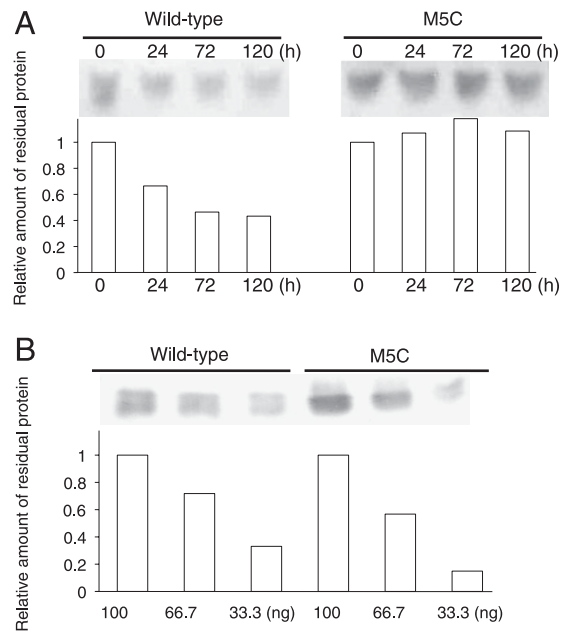


FIGURE 8. A, Western immunoblotting of wild-type Phl p 7 or M5C mutant Phl p 7 in rabbit reticulocyte lysate at several time points. B, Western immunoblotting of wild-type Phl p 7 or M5C mutant Phl p 7 at 100, 66.7, and 33.3 ng. Residual proteins were quantified using Scion imaging software.

residual protein was determined by HPLC. As a result, the ratio of residual protein of Phl p 7 against undigested sample was 9.8% and that of M5C mutant Phl p 7 was 88.2%. These results indicate that the hyperstabilization of Phl p 7 significantly increased its resistance to protease digestion.

Discussion

Recombinant human proteins have been reported to induce Ab production (12, 41, 42), and the therapeutic administration of humanized or chimeric Abs, in which the murine constant regions are replaced with human ones, has led to an anti-Ab response (43). In addition, there is extensive overlap between the induction of Abs to therapeutic mAbs between anti-humanized and anti-chimera Abs, making the advantage of humanization less obvious (43). These results indicate that the antigenicity of a protein is not always determined by the primary sequence of the protein Ag. In contrast, the data from the literature suggest that immune responses to therapeutic proteins vary within a population and even between different manufacturers of the same protein (12). Although IFN- α 2a and - β 2b have minimal sequence differences, IFN- α 2a was more immunogenic than IFN- β 2b, suggesting that there was minimal structural difference between them (6). Thus, the conformation of the protein Ag also plays a key role in the immune response.

There have been few reports on the correlations between conformational stability and immune response (18–20). In this study, we demonstrated that there were inverse and linear correlations between free-energy changes for the protein unfolding and the amount of IgG production against Ags in BALB/c mice with both HEL derivatives and ML mutants. The slopes of the HEL derivatives differed from those of the ML mutants. The HEL-specific T cell response in BALB/c mice with the H-2^d haplotype is directed toward region 11–35, in addition to region 107–116 (19). 3mut-ML has only the T cell epitope of HEL 107–116. The number of T cell epitopes would determine the difference in antigenicity for BALB/c mice. Under the circumstances, because the intercepts of the ordinates in two plots of free-energy changes for the protein unfolding against IgG production were almost identical (21–23 kcal/mol), we found the threshold of free-energy change for the protein unfolding. The threshold was adapted in hyperstabilized grass pollen allergen Phl p 7. In this study, we showed the criteria for achieving conformational stability of a protein to avoid immunogenicity under physiological conditions.

Although there has been no evaluation of whether the susceptibility to lysosomal proteolysis plays an important role in determining immunogenicity in vivo, Delamarre et al. (44) demonstrated that the immunogenicity of protein Ags in vivo can be enhanced by reducing their susceptibility to lysosomal proteolysis. Additionally, those investigators suggested that less digestible Ags may have a greater chance of surviving the digestible environment within lysosomes to gain access to the cytosol for presentation on MHC class I molecules (44). MHC class I molecules generally present peptides derived from endogenous Ags, whereas it is possible that the exogenous protein Ags with MHC class I present to CD8⁺ T cells, in a pathway called cross-presentation. Accapezzato et al. (45) suggested that lysosomotropic agents, which reduce acidification of the endocytic system and inhibit Ag degradation, increased the cross-presentation efficiency. Their findings were explained as follows: Ags are less efficiently degraded, accumulate to a greater degree in the endosomes, and are ultimately more efficiently exported into cytosol. Thus, hyperstable Phl p 7 might accumulate in the endosomes and be exported more efficiently into cytosol but not via the degradation pathway for MHC class II.

Based on information that we obtained from two homologous lysozymes, a protein with stability greater than ~20 kcal/mol would be expected to suppress IgG production. The hyperstable

Phl p 7 mutant had a stability >20 kcal/mol and significantly decreased IgG production. Consequently, this study demonstrated that the conformational stability of a protein is an immunologically dominant factor, based on the finding of the criteria for free-energy change for protein unfolding to prevent IgG production.

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Disclosures

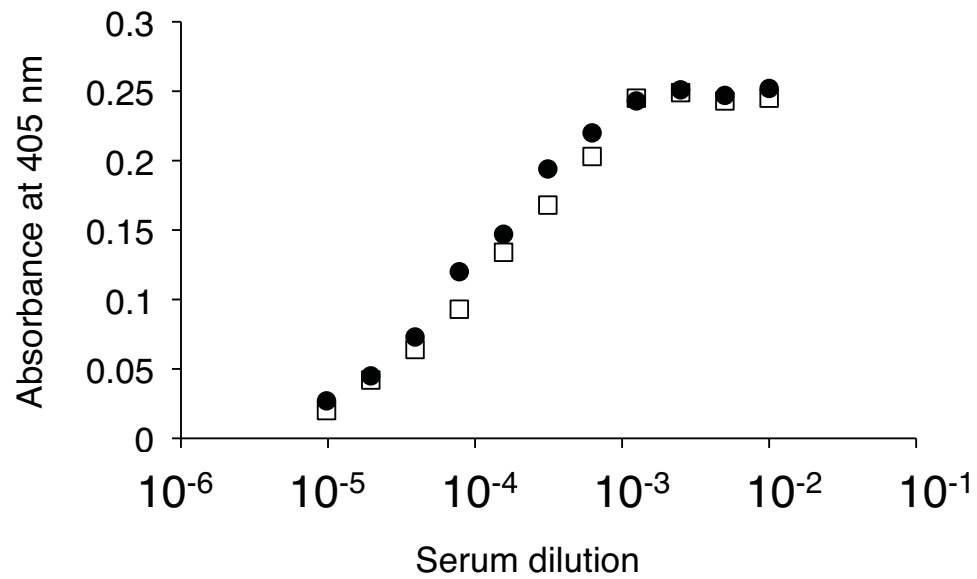
The authors have no financial conflicts of interest.

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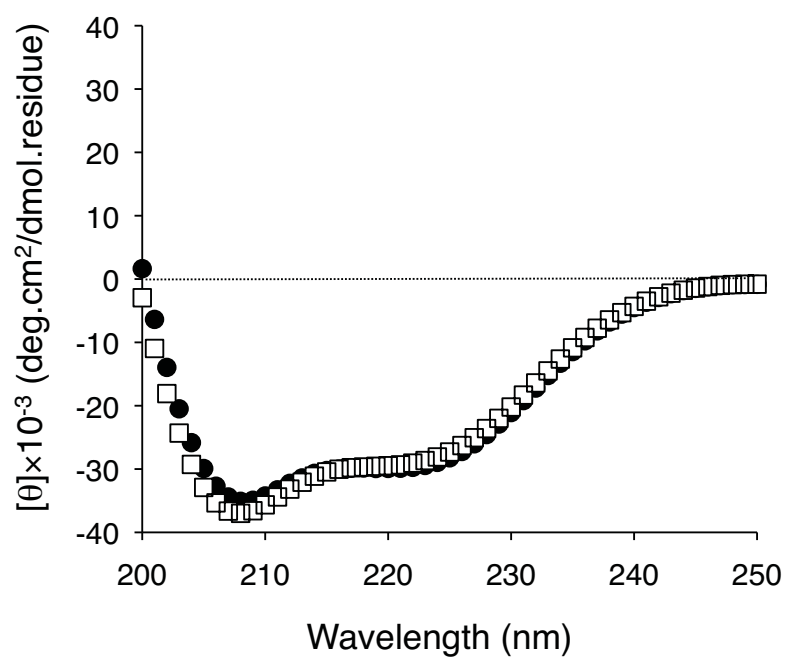
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Supplemental Figure S1



Supplemental Figure S1. Wild-type Phl p 7 (solid circles) and M5C mutant Phl p 7 (open squares) were coated onto an ELISA plate, and antibody binding levels in diluted sera were evaluated. Sera were obtained at 42 days from Balb/c immunized with wild-type Phl p 7.

Supplemental Figure S2



Supplemental Figure S2. Far-UV CD spectrum of Wild-type Phl p 7 (solid circles) and M5C mutant Phl p 7 (open squares). The protein solution is 20 mM phosphate buffer, pH 7.0 and then the CD spectrum was obtained with a Jasco-J 720 spectropolarimeter.