Antigen Stability Controls Antigen Presentation

Received for publication, May 24, 2004, and in revised form, August 3, 2004
Published, JBC Papers in Press, September 10, 2004
DOI 10.1074/jbc.M405738200

Robert Thai, Gervaise Moine, Michel Desmadril, Denis Servent, Jean-Luc Tarride, André Ménez, and Michel Léonetti
From the Département d’Ingénierie et d’Etudes des Protéines, Commissariat à l’Energie Atomique, C.E. Saclay, 91191 Gif-sur-Yvette, France, and Laboratoire de Modélisation et d’Ingénierie des Protéines, EP1089 Université de Paris-Sud, F-91405 Orsay, France

We investigated whether protein stability controls antigen presentation using a four disulfide-containing snake toxin and three derivatives carrying one or two mutations (L1A, L1A/H4Y, and H4Y). These mutations were anticipated to increase (H4Y) or decrease (L1A) the antigen non-covalent stabilizing interactions, H4Y being naturally and frequently observed in neurotoxins. The chemically synthesized derivatives shared similar three-dimensional structure, biological activity, and T epitope pattern. However, they displayed differential thermal unfolding capacities, ranging from 65 to 98 °C. Using these differentially stable derivatives, we demonstrated that antigen stability controls antigen proteolysis, antigen processing in antigen-presenting cells, T cell stimulation, and kinetics of expression of T cell determinants. Therefore, non-covalent interactions that control the unfolding capacity of an antigen are key parameters in the efficiency of antigen presentation. By affecting the stabilizing interaction network of proteins, some natural mutations may modulate the subsequent T-cell stimulation and might help microorganisms to escape the immune response.

T helper cell activation requires presentation of protein antigens (Ags) by class II major histocompatibility (MHC) molecules on the surface of antigen-presenting cells (APCs). To fit into the MHC grooves, protein Ags must possess adequate sequences that adopt an appropriate extended conformation (1, 2). In a few cases, the Ag is flexible enough to bind directly to the MHC molecule through its specific sequence (3, 4). In general, however, the protein needs to be unfolded for its MHC-specific sequence to adopt the competent binding conformation (5, 6). Unfolding in APCs, therefore, is an indispensable event that protein Ags must undergo to be presented to helper T cells (7, 8).

A number of APC parameters, which might act independently or in synergy (9), are associated with unfolding of protein Ags and may control their processing. These include a lowering of pH (10–12), a reducing activity to breakdown disulfide bonds (13–16), and endosomal/lysosomal proteases (17–19). Efficacy of these parameters depends on the intrinsic characteristics of Ags (i.e. their stability in acidic pHs (20, 21), the presence of disulfide bonds (22), the presence of protease cleavage sites (23–25), the local structural stability (26), and structural constraints associated with their tertiary (27, 28) and quaternary structures (29, 30)). Therefore, efficiency of Ag processing depends on a complex interplay between APC processing capacity and the intrinsic characteristics of an Ag, which control the unfolding capacity. In other words, the stability of a protein Ag may be a critical issue for its processing to occur efficiently.

Two types of interactions govern the structural stability of a protein. First, covalent interactions are assured by the polypeptide chain and the intramolecular disulfide bridges. Their influence on Ag presentation efficacy was previously suggested from experiments performed with HEL derivatives, which differed in their stability by the deletion of one intramolecular disulfide bond or addition of intramolecular chemical cross-links (22, 31). However, it is difficult fully to appreciate the impact of such important modifications on Ag structure. The second type of stabilizing interaction involves non-covalent contacts, which include hydrophobic interactions, van der Waals interactions, hydrogen bonds, and electrostatic interactions. It remains to be demonstrated that such non-covalent stabilizing contacts, which contribute to the stabilization of the 3D structure of a protein Ag, control presentation efficacy.

In this study, we compared the T cell stimulation capacity of four protein variants that shared similar 3D structure, biological activity, and T epitope pattern but differed in stability. The four proteins include the structurally well defined snake toxin α, a neurotoxin with four disulfide bonds (32), and three chemically synthesized derivatives that differ from toxin α by at most two substitutions introduced in the stabilizing core (L1A, L1A/H4Y, and H4Y). These mutations were anticipated to increase (H4Y) or decrease (L1A) the Ag non-covalent stabilizing interactions, H4Y being naturally and frequently observed in neurotoxins (33). Our data show that the non-covalent interaction-based stability of the Ag controls processing in APCs, T cell stimulation, and kinetics of expression of T cell determinants. Our findings suggest that natural mutations that are silent for the biological activity, structure, or T epitope pattern of a protein can alter its network of stabilizing interactions, which might affect the way it is perceived and treated by the immune system. Some microorganisms may exploit such mutations to escape the immune system.

EXPERIMENTAL PROCEDURES
Toxins—The toxin derivatives were synthesized on a 431A peptide synthesizer (Applied Biosystems, Foster City, CA) using procedures...
described previously (34). Analytical standard chemicals were from Sigma. Peptide synthesis chemicals were from Calbiochem-Novabiochem Corp. The Fmoc/troc-butyl strategy was followed using a rink amide resin (available 0.47 mmol/g) and a 20-fold excess of Fmoc. Polypeptides were cleaved from the resin, and the side-chain-protecting moieties were removed with 5% trisopropylsilane in 90% trifluoroacetic acid (TFA). Then, the peptides were precipitated with diethyl ether and solubilized in 10% acetic acid. After lyophilization, the crude materials were solubilized in 0.1% TFA and purified by HPLC on C18 reverse phase columns (Vydac, Hesperia, CA). The elution was carried out with a linear gradient of acetonitrile/water containing 0.1% TFA. Then, the four disulfide bonds were made in 100 mM phosphate buffer, pH 8.5, containing glutathione (reduced/oxidized) = 4 (mM/2 mM)). The folded proteins were subsequently purified by reversed-phase HPLC. 

Mass determination was performed on a QTRAP 2000 mass spectrometer (Micromass Ltd., Altrincham, UK).

Reduced and carboxymethyllated (RCM) toxin α and its RCM derivatives were obtained by reducing the disulfide bonds with tris(carboxyethyl)phosphine (TCEP, 5 eq) in a 50 mM phosphate buffer, pH 8, containing 6 mM urea. After 5 min of incubation, the free thiols were blocked with 10 eq of iodoacetamide. The denatured proteins were subsequently purified by reversed-phase HPLC.

Biophysical Characterization—CD measurements were performed at 37 °C with a Jasco J-715 spectropolarimeter. Protein concentrations were determined at 280 nm from CD6 Dichrograph. Spectra were recorded at 24 h at 37 °C using a Jobin-Yvon CD6 Dichrograph. Proteins were diluted in 5 mM MES/phosphate buffer and different pH values were measured.

Heat-induced unfolding was monitored as a function of temperature, at pH 7 (37 °C using a Jobin-Yvon CD6 Dichrograph). Proteins were diluted in 5 mM MES/phosphate buffer and different pH values were produced by addition of NaOH or HCl. Far-UV spectra were recorded at each pH value. The molar ellipticities at 198 nm were extracted to build secondary structure denaturation curves as a function of pH.

Susceptibility of the proteins to proteolysis was assessed using lysosomal inhibitors. The effect of inhibitors on Ag presentation was assessed in U-bottom microculture wells (Nalge Nunc International). A20 cells (10⁶/well) were cultured at 37 °C in the presence of serial dilutions of either αLIA or αH4Y and of fixed amounts of leupeptin (10 μM), pepstatin (0.1 μM), and phenylmethylsulfonyl fluoride (0.1 μM). After 16 h, cells were washed, and 5 × 10⁵ TIC9 were added per well. After 24 h at 37 °C, the supernatants were recovered and assayed for IL-2 content as described above.

Stability to Reduction and to Proteolysis by Cathespin L—Reduction was carried out by incubating each protein (0.5 μg/mL) with various concentrations of TCEP for 24 h at 37 °C in 50 mM sodium acetate buffer, pH 4.5. The samples were diluted to 1/10 in 0.1% TFA and analyzed by reversed-phase HPLC. The disappearance of the oxidized form of the toxins was followed as a function of TCEP concentrations.

RESULTS

The Selected Protein Ag Has a High Thermodynamic Stability—Various structurally unrelated proteins have been extensively studied regarding class II MHC processing and presentation. These include hen egg lysozyme, ovalbumin, ribonuclease A, and cytochrome c. To establish a framework for the comparison for our selected Ag model, a snake neurotoxin named toxin α (α), we monitored the CD ellipticity at a wavelength that is the most characteristic of the secondary structure of all these proteins as a function of temperature, at pH 7 (not shown). We found that ribonuclease A unfolds at a relatively low temperature (m.p., 57 °C), whereas hen egg lysozyme, ovalbumin, cytochrome c and toxin α unfold with higher m.p. of 72 °C, 73 °C, 72 °C and >85 °C, respectively. These proteins possess unrelated structures, distinct T cell epitopes, and differential presentation mechanisms, making it difficult to compare them directly. However, it has been shown that toxins unfold at an intermediate temperature (57 °C), which is in the range of those proteins. Therefore, we focused on the study of the structural stability of toxins, which is similar to other proteins that have been extensively studied.
difficult to identify a possible correlation (if any) between their different stabilities and presentation efficacies. To minimize the number of parameters to be considered in a comparative study, we prepared Ags of different stability but similar 3D structures and identical T-cell epitopes.

**Design and Synthesis of Three Derivatives of Toxin α—**Previous reports have shown that protein stability can be enhanced or decreased as a result of a small number of mutations (40–43). We have attempted to modulate the conformational stability of toxin α by introducing at most two substitutions in its amino acid sequence. The overall folding of this 61-residue protein consists of three major loops rich in β-sheet secondary structure once substitutions have been introduced at position 4. The three major loops protrude from a small globular core, which contains the disulfides. In this core region, we identified four buried residues that are locked in interactions with the surrounding residues. It was anticipated that shaving of the Leu1 side chain would disrupt the stabilizing interactions, whereas replacement of the imidazole ring of His4 by a phenol group would reinforce them.

Toxin α and Its Three Derivatives Share Similar Structural and Biological Properties—**The CD spectra of the three derivatives superimposed well with that of the wild-type toxin (Fig. 2A). In the far-UV, we observed the same large positive band at 198 nm and the same negative signal at 215 nm, suggesting that the predominant β-sheet secondary structure present in the native toxin has not been altered by the substitutions. The near-UV CD profiles of the wild-type toxin α and of its three derivatives also compared well, suggesting a preservation of the 3D structure (data not shown). A slight difference was observed around 280 nm in the two mutants possessing the mutation H4Y. This variation reflects the dichroic contribution of the additional aromatic residue.

Indirect information on the structure of the derivatives was derived from their ability to bind to three conformation-sensitive macromolecular targets: two toxin α-specific mAbs, Mα2–3 (46) and Mα1 (47), and the physiological target of the neurotoxins, the nicotinic acetylcholine receptor (AcChR) (36). The toxin binds to the AcChR and Mα2–3 through highly overlapping regions, which involve residues of the three loops. Mα1 binds to an opposite region of the toxin, its epitope involving residues from the N-terminal region and the large turn between loops I and II. It has been established that the N-terminal region is critically involved in the Mα1 epitope, rules out the possibility of using this Ab to probe the epitope structure once substitutions have been introduced at position 1. As shown in Fig. 2B, the binding of the antibody toxin α to AcChR was inhibited by similar amounts of the four proteins. In addition, the four toxins competed for the epitope with the mAb Mα2–3 (Fig. 2C). Therefore, the regions by which the toxin binds to the receptor and Mα2–3 were unaffected by the presence of any of the substitutions. The toxin and the derivative α H4Y were also equipotent in binding to Mα1 (Fig. 2D), indicating that this substitution had no structural effect on the Mα1 epitope, despite the close topographical proximity of position 4 (48). As a whole, the three derivatives possess structural and biological properties that are highly similar to those of the parent protein.

Native and Substituted Toxins Have Different Thermal Stabilities at Neutral and Acidic pHs—**The conformational stabil-
ity of toxin α and its three derivatives was first examined at neutral pH, by monitoring changes in CD spectra, as a function of a linear temperature gradient. Fig. 3A shows that the derivative αLIA unfolds at a temperature 15 °C lower than that at which toxin α unfolds. This result indicates that the substitution of Leu1 has impaired the conformational stability of the molecule. In contrast, the CD-derived m.p. values that characterize toxin α and the two derivatives αLIA,H4Y and αH4Y were so close to each other that they could not be determined accurately enough. Instead, they could be measured using differential scanning calorimetry. The thermograms showed clear differences between the four toxins (Fig. 3C). Asymmetry of the curves as well as the observation of an irreversible aggregation prevented an accurate calculation of the ΔH values. The qualitative thermal stability order was: αLIA (75.3 °C) < αLIA,H4Y (88.4 °C) < α (92 °C) < αH4Y (98.6 °C). Thus, at acidic and neutral pH, the conformational stability of both αH4Y and αLIA,H4Y remained virtually the same, whereas it had decreased by 6 and 12 °C for αLIA and toxin α, respectively. These two derivatives, in which m.p. was pH-sensitive, possess a histidine at position 4. Its pK value is near 5.5, making the imidazole ring protonated at pH 4.3 and not at pH 7.0. Thus, the protonated histidine in the two sensitive toxins might be responsible for their decrease in stability at acidic pH.

Native Toxin α and Its Derivatives Have Different Ag Presentation Efficacies—We compared the ability of toxin α and its three derivatives to stimulate two toxin α-specific T cell hybridomas, T1B2 and T1C9 (37). In the presence of living A20 cells, the presentation capacity followed the order αLIA > toxin α = αLIA,H4Y > αH4Y (Fig. 4, A and B), which correlated with the inverse order of thermostability of the four proteins. As observed when monitoring thermostabilities, we found that the opposite effects caused by the two individual substitutions L1A (highest degree of presentation) and H4Y (lowest presentation) were virtually compensated when introduced concomitantly (L1A/H4Y).

Next, we estimated the efficacy of Ag presentation when
toxin α and its three derivatives were previously RCM. In the presence of living A20 cells (Fig. 5A), minor differences were observed among the four unfolded proteins in their ability to stimulate the T cell hybridoma T1C9, indicating that the four toxins differ in their presentation efficacy only when they are folded. Furthermore, in the presence of fixed APCs (Fig. 5B), the four unfolded proteins were presented with the same efficacy, indicating that the ability to bind to the MHC molecule is not impaired by the substitution of the residues leucine 1 and histidine 4.

**Toxin α and Its Derivatives Require Processing for Presentation**—One mechanism that may account for the observed effects could be that some of the derivatives are flexible enough to bind to MHC class II molecules without having been processed (3, 4). We ruled out this possibility when comparing the presentation of the four proteins by fixed A20 cells. As a control, we used the peptides 32–49 and 24–41, whose presentation requires no processing (37). Fig. 4, C and D, shows that fixed APCs retained the capacity to present the two peptides but none of the four proteins.

The strongest stimulating potency of αL1A could also be related to an enhancement of its proteolysis by an aminopeptidase (50, 51). Thus, although leucine 1 is buried in the protein, its substitution with an alanine could make this position more flexible and thereby more susceptible to exo-enzymatic attack. To examine this possibility, we acetylated the amino-terminal residue of αL1A, to block the potential action of an exopeptidase and compared the stimulating potency of αL1A and N-acetylated αL1A. As shown in Fig. 4, E and F, the two derivatives are equally potent in stimulating T cells, indicating that an amino-terminal proteolysis, if any, does not affect the presentation of αL1A.

**Kinetics of Expression of T Cell Epitopes on the Surface of APCs**—We compared the kinetics of presentation of the two T cell epitopes, by pulsing A20 cells for different periods of time with toxin α and the three toxin derivatives. At various intervals after the Ag pulse, APCs were washed and fixed, and T1B2 (Fig. 6A) or T1C9 (Fig. 6B) was added to the probe presentation. The results show that the two T cell epitopes were expressed more rapidly when αL1A was used. They were presented after a longer incubation period when toxin α and αL1A,H4Y were used. The greatest delay in the appearance of the T cell determinants was observed with αH4Y. Therefore, the kinetics of expression of the T cell determinant are increased when the conformational stability of the protein decreases.

**Susceptibility of Toxin α and Its Derivatives to pH Decrease, TCEP Reduction, and Proteolysis**—The three intracellular conditions that may participate in Ag processing are a decrease in pH, a reducing activity, and a proteolytic activity. We investigated whether the efficacy of these three events may be influ-

---

**Fig. 3.** **Toxin α and its derivatives display different stabilities.** The apparent fraction of native protein is shown as a function of temperature. The thermal denaturation curves of all proteins were determined by monitoring the change in the characteristic β-sheet CD band at 198 nm at neutral pH (A) or at pH 4.3 (B) in 5 mM MES/phosphate buffer. Differential scanning calorimetry thermograms of the four proteins were recorded in the same experimental conditions in 5 mM phosphate buffer, pH 7, at 1.5 K/min scan rate (C).
enced by the differential stability of the four Ags.
To examine the effect of decreasing pH on the unfolding of the four proteins, we monitored the CD spectra of each protein as a function of a linear pH gradient, at 37 °C. Fig. 7A shows that the folding of the three most stable Ags (i.e. α/H9251 H4Y, toxin α/H9251 and α/H9251 L1A,H4Y) remained unaffected between pH 7 and 2. An onset of unfolding of the derivative α/H9251 L1A occurred just below pH 3.5, which was followed by a clear denaturation down to about pH 2. Therefore, the toxin and its derivatives all remain in the same native-like conformation down to pH 3.5.
Reduction of the disulfide bonds of the four Ags was determined at acidic pH (4.3) by measuring the disappearance of the native toxin after a 2-h incubation at 37 °C, using the low pH-compatible reducing reagent TCEP (52). As shown in Fig. 7B, the disulfides of the four molecules are reduced with comparable efficacy, indicating that the redox potential of the disulfides is similar in the four proteins. To examine whether the wild-type and derivatized toxins behaved differently toward proteolysis, we subjected them to the action of cathepsin L, one of the enzymes known to be critical in Ag processing (53), in slightly reducing conditions (0.8 eq of TCEP (mol/mol) compared with the protein concentration). Fig. 7D shows that the derivative αL1A was almost completely degraded in 4 h, giving rise to a variety of well defined fragments, which all eluted before the undegraded toxin. A partial degradation was observable for α/H9251 L1A (Fig. 7C) and α/H9251 L1A,H4Y (Fig. 7E), but after 4 h of incubation, a large proportion of native toxin clearly remained intact. Finally, virtually no degradation of α/H9251 H4Y (Fig. 7F) was observed after the same incubation time. It was clear that resistance to hydrolysis by cathepsin L correlated with protein stability.

Effect of Ag Stability on Ag Degradation and Presentation by APCs—We examined whether a variation in Ag stability is associated with a differential half-life in APCs. To investigate this, αL1A and αH4Y were tested competitively for their capacity to be degraded by APCs. To negate the fate of degraded derivatives and not of the released fragments because the proteolytic products have short half-lives until they are captured by class II molecules (54–56). The two derivatives

![Fig. 4](image_url)  
**Fig. 4.** Toxin α and its derivatives differ in their Ag presentation efficacy. Serial dilutions of Ag were incubated with either T1B2 or T1C9 (5 × 10⁴ hybridomas/well) in the presence of living (A and B) or fixed (C and D) A20 cells (5 × 10⁴ cells/well). After 24 h at 37 °C, supernatants were collected, and IL-2 secretion was determined by cytotoxic T cell line assay. The possible influence of aminopeptidase proteolysis on the stimulating potency of αL1A was assessed with αL1A and acetylated αL1A (Ac αL1A) incubated with A20 cells and T1B2 (E) or T1C9 (F).

![Fig. 5](image_url)  
**Fig. 5.** Toxin α and its derivatives have similar Ag presentation efficacy when they are previously denatured and carboxamidomethylated. Serial dilutions of reduced and carboxamidomethylated (RCM) Ag were incubated with T1C9 (5 × 10⁴ hybridomas/well) in the presence of living (A) or fixed (B) A20 cells (5 × 10⁴ cells/well). After 24 h at 37 °C, supernatants were collected, and IL-2 secretion was determined by cytotoxic T cell line assay.
were first labeled with tritium by a method that makes labeled proteins virtually indistinguishable from the native ones (38). The tritiated derivatives, which have the same specific activity, were then incubated with APCs, recovered from cell lysates by immunoprecipitation, and subjected to SDS-PAGE. Unprocessed native [3H]αL1A and [3H]αH4Y were also included in the gels as a control. The gel was blotted onto a membrane that was subjected to radioactivity measurement using a highly sensitive β-imager. As shown in Fig. 8A, radioactivity was only observed at the level of proteins that migrated with the expected molecular mass (6.8 kDa) of undegraded toxins (lanes 1 and 2 for unprocessed [3H]αL1A; lanes 6 and 7 for unprocessed [3H]αH4Y). The most striking difference resides in the very low level of tritium in lane 3, which corresponds to the migration of [3H]αL1A. More precisely, 2982 disintegrations have accumulated in this lane. In contrast, a higher proportion of radioactivity (19,895 disintegrations) accumulated in lane 5, which corresponds to migration of [3H]αH4Y. Therefore, incubation with APCs has selectively caused the least stable derivative to vanish.

We examined whether the presence of inhibitors could be stow on αL1A the capacity to stimulate Ag presentation as efficiently as αH4Y in the absence of inhibitors. We incubated A20 cells with αL1A and αH4Y in the presence or absence of inhibitors. APCs were then washed and presentation was probed with T1C9. Fig. 8B shows that in the presence of 0.1 μM PMSF, 0.1 μM pepstatin, and 10 μM leupeptin, the presentation of αL1A became comparable with that observed with αH4Y in the absence of inhibitors. The presence of the same concentration of inhibitors had no effect on the presentation efficacy of the most stable Ag, but in the presence of a 10-fold higher inhibitor concentration, the presentation efficacy was clearly affected for both Ags. As a control, we determined that the enzyme inhibitors did not interfere with the expression of class II molecules by showing that efficacy of presentation of peptide 24–36 to T1C9 remained unaffected in the presence of the inhibitors (data not shown). Therefore, Ag stability controls processing efficacy in APCs, which in turn modulate Ag presentation.

DISCUSSION

We have explored the possibility that variations in conformational stability of a protein Ag, as induced by introduction of subtle changes in the network of non-covalent stabilizing interactions, could affect the capacity of an Ag to stimulate T cells. We used a snake neurotoxin named toxin α and three synthetic variants (34), which all displayed differential stability. The substitutions were introduced at positions 1 and/or 4 in the stabilizing core region, but distant from both the toxin α-specific T cell epitopes recognized in the H-2d haplotype (localized in the region 24–49) and its flanking regions (57, 58). We carefully checked that the four available Ags (toxin α, αL1A, αH4Y, and αL1A,αH4Y) possessed the same overall 3D structure. This was inferred from both their virtually identical CD spectra and their highly similar ability to bind to three conformation-sensitive targets, two mAbs and AcChR. Despite their remarkably similar 3D structures, the four Ags displayed differential stability characterized by m.p. values covering a range of about 25 °C, at both neutral and acidic pHs.

We have first demonstrated in vitro that the efficacy of T-cell stimulation of Ags correlated inversely with their conformational stability. The more stable the Ag, the less efficient it was at stimulating T cells. This stimulation does not result from a direct binding of the Ags to the class II MHC molecules expressed on the surface of the APCs (3, 4), because we found no presentation when using fixed APCs. Therefore, processing was required for the four Ags to stimulate T cells. Experiments with pulsed APCs have revealed marked differences in the kinetics of stimulation between the four Ags, revealing distinct rates of expressions of the two toxin-T-cell determinants on the surface of APCs, suggesting differential processing efficacy. This proposal is further supported by our observation that the half-life of two labeled Ags in APCs depends on their stability. More precisely, we have observed that after 16 h of incubation with A20 cells, the proportion of labeled αH4Y, the most stable derivative, was ~7-fold higher in the cell lysates compared with that of labeled αL1A, the least stable derivative.

It could be argued that variations in stimulation efficacy could have origins other than the intrinsic differential stability of the four Ags. Thus, we wondered whether the substitutions had generated new T cell epitopes in the Ag that would compete with the natural ones, as observed in other cases (59, 60). To discount this possibility, we synthesized the peptides 1–15L1A, 1–15H4Y, and 1–15H4Y and showed that none of them had generated new T cell epitopes in the Ag that would compete with the natural ones, as observed in other cases (59, 60). To rule out this possibility by showing that an Ag derivative selectively acetylated on its NH₂-terminal group stimulates T-cells with the same efficacy as the wild-type Ag. Having disclosed these possible scenarios, we concluded that the conformational stability of an Ag may be the parameter that controls its T cell stimulation efficacy, as a result of a differential ability to be processed by APCs.
APCs possess various elements that contribute to the processing of Ags and that might act independently or in synergy (9). These include a medium whose pH progressively decreases to about 4.5, a machinery to reduce disulfide bonds, and a variety of proteolytic enzymes. Although the four Ags were characterized by differential stability, they all resisted unfolding down to pH 3.5, suggesting that their processing is not directly affected by the intracellular pH. The four toxins were also similarly susceptible to disulfide reduction by dithiothreitol at pH 7.0 (data not shown) and TCEP at pH 4.3, indicating that they have a similar redox potential. However, this does not preclude the possibility that disulfide-reducing enzymes acting at low pHs, such as γ-interferon-inducible lysosomal thiol reductase (13, 14), may reduce the four Ags with different efficac-
cies. Two lines of evidence suggest that a critical parameter that controls processing efficacy of the four Ags is the susceptibility to enzymatic proteolysis. First, cathepsin L was able to proteolysate at pH 4.3 the native and derivatized toxins with differential efficiencies. The more stable the toxin, the less susceptible it was to proteolysis by cathepsin L. This observation was not specifically related to the enzymatic activity of cathepsin L, because a similar pattern of stability-related differential susceptibilities was observed when the four Ags were submitted to proteolysis by high concentration (25 mg/l) of Pronase E (data not shown). Second, the presentation efficacy of the least stable derivative became comparable with that of the most stable derivative upon addition of an appropriate concentration of enzyme inhibitors. Therefore, we conclude that Ag presentation efficacy results from differential proteolytic efficiencies of the four Ags.

Because they occurred in the Ag stabilizing core, at most two substitutions (Leu1Ala and His4Tyr) suffice to cause marked differential stability. Thus, 80% of Leu1 is buried in the toxin, establishing several van der Waals contacts, locking loops 1 and 2 and maintaining the C-terminal ring in contact with the hydrophobic core of the toxin. Shaving the side chain of Leu1 was anticipated to create a cavity in this network of interactions and to destabilize the toxin architecture (40, 61). Introduction of an alanine indeed caused a substantial stability decrease. Although His4 is polar, it is also buried in the core region. Its replacement by the more hydrophobic tyrosine was anticipated to create new interactions and to increase the toxin’s conformational stability. This variant was indeed more stable than the native toxin. The differential stability caused by these substitutions sufficed to increase the susceptibility of the toxin to proteolysis, perhaps by increasing the flexibility of the toxin structure around some proteolytic sites. Therefore, a small number of mutations may suffice to modify subtly the network of non-covalent stabilizing interactions of an Ag, which, in turn, may substantially affect its processing and its T cell-stimulating efficacy.

Therefore, we have provided experimental evidence that stability can be a predominant parameter in the control of Ag processing. We may now look differently at mutations that occur in proteins either naturally, such as H4Y, or that are human-designed, such as L1A, and that cause no change in their biological activity, 3D structure, or T epitope pattern. Indeed, when such apparently silent mutations occur in protein-stabilizing cores, they could affect protein stability and hence T-cell response. When such mutations occur naturally, they might cause substantial modulations in immune responses to proteins, and we wonder whether microorganisms exploit such features for immune escape.

Acknowledgments—We gratefully acknowledge F. Beau for assistance in β-imager experiments. We thank Drs. S. Zinn-Justin and B. Gilquin for providing unpublished data and for their advice on the selection of the derivatives of toxin α.

REFERENCES
1. Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1993) Nature 364, 33–39
2. Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1994) Nature 368, 215–221
3. Kunnela, H. A., Weber, D. A., Moore, J. C., Westerman, L. E., and Jensen, P. E. (1997) Mol. Immunol. 34, 471–480
4. Lee, P., Matsueda, G. R., and Allen, P. M. (1988) J. Immunol. 140, 1063–1068
5. Jensen, P. E. (1990) J. Immunol. 150, 3347–3356
6. Sette, A., Adorini, L., Colon, S. M., Buus, S., and Grey, H. M. (1989) J. Immunol. 143, 1265–1267
7. Allen, P. M., and Unanue, E. R. (1984) Am. J. Anat. 170, 483–490
8. Streicher, H. Z., Berkower, I. J., Buus, M., Gurd, F. R., and Berzdorff, J. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6831–6835
9. Watts, C. (2001) Curr. Opin. Immunol. 13, 26–31
10. Chesnut, R. W., Colon, S. M., and Grey, H. M. (1982) J. Immunol. 128, 1764–1768
11. McCoy, K. L., Miller, J., Jenkins, M., Ronchese, F., Germain, R. N., and Schwartz, R. H. (1989) J. Immunol. 143, 29–38
12. Ziegler, H. K., and Unanue, E. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 79, 175–178
13. Arunachalam, B., Phan, U. T., Geuze, H. J., and Cresswell, P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 745–750
14. Maric, M., Arunachalam, B., Phan, U. T., Dong, C., Garrett, W. S., Cannon, K. S., Alfonso, C., Karlsson, L., Flavell, R. A., and Cresswell, P. (2001) Science 294, 1361–1365
15. Jensen, P. E. (1991) J. Exp. Med. 174, 1121–1130
16. Collins, D. S., Unanue, E. R., and Harding, C. V. (1991) J. Immunol. 147, 4054–4059
17. Manoury, B., Hewitt, E. W., Morrice, N., Dando, P. M., Barrett, A. J., and...
Antigen Stability and Antigen Presentation

Watts, C. (1998) Nature 396, 695–699
18. Plüger, E. B., Boes, M., Alfonso, C., Schröter, C. J., Kalbacher, H., Ploegh, H. L., and Driessen, C. (2002) Eur. J. Immunol. 32, 467–476
19. Villadangos, J. A., and Ploegh, H. L. (2000) Immunity 12, 233–239
20. Chianese-Bullock, K. A., Russell, H. I., Moller, C., Gerhard, W., Monaco, J. J., and Eisenlohr, L. C. (1998) J. Immunol. 161, 1999–1997
21. Eisenlohr, L. C., Gerhard, W., and Hackett, C. J. (1988) J. Immunol. 141, 1870–1876
22. So, T., Ito, H. O., Koga, T., Watanabe, S., Ueda, T., and Imoto, T. (1997) J. Biol. Chem. 272, 32136–32140
23. Antoniou, A. N., Blackwood, S. L., Mazzeo, D., and Watts, C. (2000) J. Immunol. 12, 391–398
24. Manoury, B., Mazzeo, D., Fugger, L., Viner, N., Ponsford, M., Streeter, H., Mazza, G., Wraith, D. C., and Watts, C. (2002) Nat. Immunol. 3, 169–174
25. Schneider, S. C., Ohmen, J., Fodick, L., Gladstone, B., Guo, J., Ametani, A., Sercarz, E. E., and Deng, H. (2000) J. Immunol. 165, 20–23
26. Carmicle, S., Dai, G., Steede, N. K., and Landry, S. J. (2002) J. Biol. Chem. 277, 155–160
27. Glimcher, L. H., Schroer, J. A., Chan, C., and Shevach, E. M. (1983) J. Immunol. 131, 2686–2674
28. Ametani, A., Sakurai, T., Katakura, Y., Kubara, S., Hirakawa, H., Hosi, T., Dosako, S., and Kaminogawa, S. (2003) BioSci. Biotechnol. Biochem. 67, 1507–1514
29. Janssen, R., Wauben, M. H., and Tommassen, J. (1996) Int. Immunol. 8, 829–836
30. Rouss, N., Christophe, S., Rousseau, F., Bellet, D., Guillet, J. G., and Bidart, J. M. (1993) J. Immunol. 150, 782–792
31. So, T., Ito, H., Hirata, M., Ueda, T., and Imoto, T. (2001) Immunology 104, 259–268
32. Zinn-Justin, S., Roumestand, C., Gilquin, B., Bontems, F., Menez, A., and Toma, F. (1992) Biochemistry 31, 1133–1145
33. Endo, T., and Tamiya, N. (1991) in Snake Toxins (Harvey, A. L., ed.), pp. 3597–3603
34. Mourier, G., Servent, D., Zinn-Justin, S., and Menez, A. (2000) Protein Eng. 13, 217–225
35. Alexander, P., Fahnestock, S., Lee, T., Orban, J., and Bryan, P. (1992) Biochemistry 31, 3097–3093
36. Ishikawa, Y., Menez, A., Hori, H., Yoshida, H., and Tamiya, N. (1977) Toxicon. 15, 477–488
37. Maillet, B., Cotton, J., Mourier, G., Léonetti, M., Leroy, S., and Menez, A. (1993) J. Immunol. 150, 5270–5280
38. Menez, A., Morgat, J., Fromageot, P., Ronseray, A., Boquet, P., and Changeux, J. (1971) FEBS Lett. 17, 323–325
39. Bonifacino, J. S., Dell’Angelie, R. C., and Springer, T. A. (2001) in Current Protocols in Immunology (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Sharpe, E. M., Strober, W. A., eds) Vol. 2, Chapter 8.3, John Wiley & Sons, Inc., New York
40. Akasako, A., Haruki, M., Osbatake, M., and Kanaya, S. (1997) J. Biol. Chem. 272, 10358–10365
41. Fagiani, C. O. (1995) Biochim. Biophys. Acta 1232, 1–14
42. Matsunura, M., Yasumura, S., and Aiba, S. (1986) Nature 323, 356–358
43. Stearman, R. S., Frankel, A. D., Freire, E., Liu, B. S., and Pabo, C. O. (1988) Biochemistry 27, 7571–7574
44. Léonetti, M., Pillet, L., Maillet, B., Lanthanh, H., Frachon, P., Couderc, J., and Menez, A. (1990) J. Immunol. 145, 4214–4221
45. Kanaya, S., Osbatake, M., Nakamura, H., and Ikehara, M. (1993) J. Biotechnol. 28, 117–136
46. Trémeau, O., Boulain, J. C., Couderc, J., Fromageot, P., and Menez, A. (1986) FEBS Lett. 208, 236–240
47. Boulain, J. C., Menez, A., Couderc, J., Faure, G., Liacopoulos, P., and Fromageot, P. (1982) Biochemistry 21, 2910–2915
48. Zinn-Justin, S., Roumestand, C., Dreyf, P., Menez, A., and Toma, F. (1993) Biochemistry 32, 6884–6891
49. Mellman, I., Fuchs, R., and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700
50. Larsen, S. L., Pedersen, L. O., Buus, S., and Stryhn, A. (1996) J. Exp. Med. 184, 183–189
51. Nelson, C. A., Vidavsky, I., Viner, N. J., Gross, M. L., and Unanue, E. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 628–633
52. Ruegg, U. T., and Rudinger, J. (1977) Methods Enzymol. 47, 111–116
53. Hsieh, C. S., deRos, P., Honey, K., Beers, C., and Rudensky, A. Y. (2002) J. Immunol. 168, 2618–2625
54. Castellino, F., Zappacosta, F., Coligan, J. E., and Germain, R. N. (1998) J. Immunol. 161, 4048–4057
55. Davidson, H. W., Reid, P. A., Lanza, A., and Watts, C. (1991) Cell 67, 105–116
56. Denermeyer, D. L., and Allen, P. M. (1989) J. Immunol. 142, 1063–1068
57. Dai, G., Carmicle, S., Steede, N. K., and Landry, S. J. (2002) J. Biol. Chem. 277, 161–168
58. Moudgil, K. D., Sercarz, E. E., and Greul, I. S. (1998) Immunol. Today 19, 217–220
59. Perkins, D. L., Berriz, G., Lamthanh, H., Frachon, P., Couderc, J., and Menez, A. (1998) FEBS Lett. 4214–4221
60. Wang, Y., Smith, J. A., Gefter, M. L., and Perkins, D. L. (1992) J. Immunol. 148, 3034–3041
61. Buckle, A. M., Cramer, P., and Fersht, A. R. (1996) Biochemistry 35, 4298–4305