Metastability in the Inhibitory Mechanism of Human α₁-Antitrypsin*

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Metastability of the native form of proteins has been recognized as a mechanism of biological regulation. The energy-loaded structure of the fusion protein of influenza virus and the strained native structure of serpins (serine protease inhibitors) are typical examples. To understand the structural basis and functional role of the native metastability of inhibitory serpins, we characterized stabilizing mutations of α₁-antitrypsin in a region presumably involved in complex formation with a target protease. We found various unfavorable interactions such as overpacking of side chains, polar-nonpolar interactions, and cavities as the structural basis of the native metastability. For several stabilizing mutations, there was a concomitant decrease in the inhibitory activity. Remarkably, some substitutions at Lys-335 increased the stability over 6 kcal mol⁻¹ with simultaneous loss of activity over 30% toward porcine pancreatic elastase. Considering the location and energetic cost of Lys-335, we propose that this lysine plays a pivotal role in conformational switch during complex formation. Our current results are quite contradictory to those of previously reported hydrophobic core mutations, which increased the stability up to 9 kcal mol⁻¹ without any significant loss of activity. It appears that the local strain of inhibitory serpins is critical for the inhibitory activity.

Facile conversion of the metastable native structure of proteins into an alternative more stable form, accompanying the execution of their functions, has been recognized as a mechanism of biological regulation. The energy-loaded structure of the fusion protein of influenza virus (1), the strained native structure of plasma serpins (serine protease inhibitors) (2), and possibly the surface glycoprotein of human immunodeficiency virus (HIV)³ (3) are typical examples. The native strain of serpins is considered to be crucial to their physiological functions, such as plasma protease inhibition (2, 4), hormone delivery (5), Alzheimer filament assembly (6, 7), and extracellular matrix remodeling (8). The inhibitory serpins, which include α₁-antitrypsin (α₁AT), antithrombin III, α₁-antichymotrypsin, and plasminogen activator inhibitor-1, serve as a good model system to study the native metastability; several crystal structures of both the strained native (9–13) and the relaxed cleaved forms (14–16) are available. In addition, the inhibitory activity that presumably is related to the native metastability is easy to assay.

The serpin structure is composed of three β-sheets and several α-helices (Fig. 1). Upon binding a target protease, the reactive center loop of inhibitory serpins is thought to be inserted into the major β-sheet, A sheet, to form a very stable complex between the inhibitor and the protease (17, 18). Various biochemical (19, 20) and structural (21–23) studies suggest that the loop insertion is necessary for the formation of a stable complex but not sufficient to confer inhibitory activity. Instead, the rate of loop insertion is thought to be critical for inhibitory function. The inhibition process of serpins can be described as a suicide substrate mechanism (17), in which serpins, upon binding with proteases, partition between cleaved serpins and stable serpin-enzyme complexes in a ratio represented by the stoichiometry of inhibition (SI: number of moles of inhibitors required to completely inhibit 1 mol of a target protease). The SI values of most inhibitory serpins are close to one for cognate target proteases. Retardation of the loop insertion, however, would alter the partitioning between the inhibitory and substrate pathways in such a way that the SI value would increase since the SI value is defined by 1 + k_{substrate}/k_{inhibition} (17, 18, 20). It is conceivable that the energy loaded in the strained native structure of serpins is utilized for the facile loop insertion.

To understand the structural basis of the loaded energy in the native structure, we have been characterizing stabilizing amino acid substitutions of α₁AT, a prototype inhibitory serpin. We previously reported that decrease in the size of side chains at the hydrophobic core of α₁AT (Fig. 1, blue) confers increased stability (24). That was quite unusual, as a decrease in size inside the hydrophobic core usually yields a cavity that causes loss of stability (25). We proposed that side chain locking in the native α₁AT prohibits rearrangement of the side chains for maximal packing (24). Such structural defects are likely to be the basis of the native strain in the inhibitory serpins. If the energy loaded in the strained native structure of serpins is utilized for the inhibitory function, mutations that decrease the loaded energy should also decrease the activity. In the present study, we tested this concept of the native strain. We searched for mutations that increased the stability and simultaneously affected the inhibitory activity. We characterized stabilizing amino acid substitutions of α₁AT in a region that is presumably involved in the conformational change for the insertion of the reactive center loop during the inhibitory complex formation: strands 3 and 5 of A sheet (s3A and s5A), helix F (hF), and the connecting loop (Fig. 1, purple). Characterization of the mutant proteins and structural examination of the mutation sites revealed various unusual interactions as the structural basis of the native metastability. Functional analyses provide direct

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The abbreviations used are: HIV, human immunodeficiency virus; α₁AT, α₁-antitrypsin; PEG, polyethylene glycol; SI, stoichiometry of inhibition.
evidence that the energy loaded in the native inhibitory serpins is utilized for the biological activity.

EXPERIMENTAL PROCEDURES

Recombinant α1AT Proteins—Plasmids for α1AT expression in Escherichia coli, and the refolding and purification of recombinant α1AT protein were described previously (26). Concentrations of α1AT were determined in 6 M guanidine hydrochloride using a value of 0.15 mg/ml. The refolding and purification of recombinant α1AT were screened after heat treatment of cell lysates at 60 °C for 1 h as described previously (26). Substitutions at specific sites were introduced by using degenerative oligonucleotides (24), and thermostable mutations of α1AT were screened after heat treatment of cell lysates at 60 °C for 1 h as described previously (26). Substitutions at specific sites were generated by oligonucleotide-directed mutagenesis.

Urea-induced Equilibrium Unfolding Transition—Equilibrium unfolding as a function of urea was monitored by fluorescence spectroscopy (λex = 280 nm and λem = 360 nm, excitation and emission slit widths = 5 nm for both). Details of which were described previously (24, 26). The buffer was 10 mM phosphate, 160 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 6.5, and the protein concentration was 10 μg/ml. The native protein was incubated in the buffer containing various concentrations of urea at 25 °C. Experimental data of the fluorescence measurement were fitted to a two-state unfolding model.

TABLE I

| Mutations | Site | ΔΔG | Relative activity |
|-----------|------|-----|------------------|
| Stability increase correlates with activity loss | | | |
| K335G (A) | s5A | 6.8 | 0.68 |
| K335A* (A) | s5A | 6.5 | 0.56 |
| K33T (A) | s5A | 3.4 | 0.77 |
| F189V (V) | s3A | 1.9 | 0.97 |
| F189V (V) | s3A | 1.9 | 0.95 |
| K331M (K) | s5A | 1.2 | 0.85 |
| T165S (T) | hF | 0.6 | 0.93 |
| L172V (R) | thFs3A | 0.3 | 1.02 |
| L172A* (R) | thFs3A | 1.5 | 0.92 |
| K174T (Q) | thFs3A | 0.3 | 0.95 |
| Activity loss without significant stability increase | | | |
| K168I (I) | thFs3A | 0.6 | 0.85 |
| K168A* (I) | thFs3A | 0.1 | 0.73 |
| I169V (I) | thFs3A | 0.0 | 0.73 |
| Stability increase without significant activity loss | | | |
| A185V (V) | s3A | 4.0 | 0.95 |
| G164V (Q) | hF | 1.9 | 1.03 |
| K163T (S) | hF | 1.5 | 1.12 |

* One-letter codes for amino acids were used. Mutations are grouped according to the relation between stability and activity loss. Ovalbumin sequences at the corresponding sites (2) are shown in parentheses.

26. Location of the mutation was designated according to Ref. 2.

5. From ΔΔG × 3.1 (average m value) in kcal mol−1. The experimental errors are ±0.16 kcal mol−1.

6. Relative activity against porcine pancreatic elastase is indicated as the ratio of the wild-type SI value over the mutant value. The experimental errors are ±0.06 in all cases.

7. Mutations, which require double base-pair substitutions, were produced by site-directed mutagenesis.

RESULTS AND DISCUSSION

Molecular Properties of Mutant α1AT

We found various stabilizing single amino acid substitutions at over 10 sites in the target region (Fig. 1). The conformational stability of representative mutant α1AT was measured by equilibrium unfolding in the presence of urea in which changes in intrinsic tryptophan fluorescence intensity were monitored. The changes in free energy of stabilization (ΔΔG) of the mutant proteins are summarized in Table I. For many of the sites, additional substitutions were made by introducing alanine, among which stabilizing mutations were further characterized (Table I). One remarkable result is that K335A and K335G mutations showed a profound effect on stability (Fig. 2A), shifting the midpoint of the unfolding transition from 1.8 to 3.9 μM and 4.0 μM urea, respectively, which resulted in the increase of stability over 6 kcal mol−1. This is the biggest increase in stability of α1AT by a single amino acid substitution studied so far. The effect of the mutations on α1AT on the inhibitory activity was examined by determining the SI values against a target protease. The SI values of K335A and K335G α1AT, which showed dramatic increase in stability (Fig. 2A), were increased substantially (Fig. 2B). The SI value of K168A, which showed little increase in stability (Fig. 2B), was also increased (Fig. 2B). The changes in SI value were due to the alteration of the partitioning between the inhibitory and substrate path.
was mixed with varying amounts of porcine pancreatic protease. A constant concentration of the protease

\[ \text{activity change was plotted as the function of stability increase} \]

was measured with 1 mM urea solution containing 10 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol (final pH, 6.5) for 8 h at 25 °C. The residual protease activity was measured with 1 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide as a substrate.

\[ \text{increase by K335A and K335G mutations compared with wild-type αAT (Fig. 3), indicating that there was a shift in partitioning toward substrate pathway in mutant proteins. The effects of other stabilizing mutations on inhibitory activity were determined and the results are summarized in Table I.} \]

Correlation between Loaded Energy and Inhibitory Activity

If the loaded energy in the native serpins is utilized for inhibitory complex formation, there should be a correlation between stability increase and loss of inhibitory activity. The activity change \( \Delta F \) was plotted as the function of stability increase for each mutant protein (Fig. 4), and the mutations were grouped into three classes according to their properties (Table I). For group I mutations (top group in Table I), there seems to be a correlation between the increase in conformational stability and the loss in inhibitory activity (Fig. 4, circles; Fig. 1, yellow beads). Remarkably, the substitutions at Lys-335, K335A and K335G, increased the stability over 6 kcal mol\(^{-1}\) with concomitant losses of activity over 30% toward porcine pancreatic elastase. These results suggest that the loaded energy is related to the inhibitory function at Lys-335, and possibly at other sites. For the group II substitutions (middle group in Table I), K168A, K168I, or I169V barely increased the conformational stability but affected inhibitory activity sub-

\[ \text{mutant} \quad \alpha_{\text{AT},} \quad \text{A}, \quad \text{urea-induced equilibrium unfolding transition. Unfolding transitions were measured by the increase in fluorescence emission intensity at 360 nm (λex = 280 nm). Samples were equilibrated in each urea solution containing 10 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol (final pH, 6.5) for 8 h at 25 °C. The protein concentration was 10 μg/ml.} \]

\[ \text{mutant} \quad \alpha_{\text{AT}}, \quad \text{A}, \quad \text{wild-type; } \Delta, \text{K168A; } K335A; \quad K335G. \]

\[ \text{FIG. 2. Molecular properties of K335A, K335G, and K168A mutant } \alpha_{\text{AT}} \quad \text{A, urea-induced equilibrium unfolding transition. Unfolding transitions were measured by the increase in fluorescence emission intensity at 360 nm (λex = 280 nm). Samples were equilibrated in each urea solution containing 10 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol (final pH, 6.5) for 8 h at 25 °C. The protein concentration was 10 μg/ml.} \]

\[ \text{mutant} \quad \alpha_{\text{AT}}, \quad \text{A}, \quad \text{wild-type; } \Delta, \text{K168A; } K335A; \quad K335G. \]

\[ \text{FIG. 3. Mutational effect on the formation of } \alpha_{\text{AT}}\text{-protease complex. The wild-type and Lys-335 mutant } \alpha_{\text{AT}} \text{ proteins were incubated with porcine pancreatic elastase at designated molar ratios (I/E), shown on the top of the figure, of } \alpha_{\text{AT}} \text{ to protease. Samples were incubated at 37 °C for 10 min in 30 mM phosphate, 160 mM NaCl, 0.1% PEG 6000, and 0.1% Triton X-100, pH 7.4. The formation of SDS-resistant } \alpha_{\text{AT}}\text{-protease complex was analyzed on 10% SDS-polyacrylamide gel electrophoresis. 5 μg of } \alpha_{\text{AT}} \text{ was loaded on each lane except lane Ez. Lanes: } \alpha_{\text{AT}}, \quad \text{A, protease incubated without porcine pancreatic elastase; wt, wild-type } \alpha_{\text{AT}} \text{ protein incubated with porcine pancreatic elastase; G, K335 mutant } \alpha_{\text{AT}} \text{ protein incubated with porcine pancreatic elastase; Ez, porcine pancreatic elastase (1 μg is loaded). Cp, inhibitor-protease complex; N, native } \alpha_{\text{AT}}; \quad \text{Cl, cleaved } \alpha_{\text{AT}}; E, \text{ porcine pancreatic elastase.} \]

\[ \text{FIG. 4. The relation between stability increase and activity loss of the mutant } \alpha_{\text{AT}} \text{ obtained in this study. Activity loss against porcine pancreatic elastase by each mutation was calculated as: } \text{A, relative activity of the mutant protein toward porcine pancreatic elastase. } \alpha_{\text{AT}}, \quad \text{A, wild-type; } \Delta, \text{K168A; } K335A; \quad K335G. \]

\[ \text{ΔAG (kcal mol}^{-1}\text{)} \]

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\[ \text{ΔAG (kcal mol}^{-1}\text{)} \]
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stastically (Fig. 4, triangles; Fig. 1, orange beads). It seems, however, that for these mutations the loss of activity is not due to an increase in conformational stability of the native form per se but to a decrease in the stability of the serpin-protease complex. Such mutations can be obtained in our initial screening for thermostable variants, because the screening is based on enhanced kinetic stability against aggregation (26). It has been suggested that the mechanism of heat-induced aggregation of α1AT is loop-sheet polymerization in which the reactive center loop of one molecule is inserted into A sheet of another (30). For the group III mutations (bottom group in Table I), K163T, G164V, or A183V, the activity of α1AT was less affected than expected from the increase in stability (Fig. 4, green squares; Fig. 1, green beads). Unlike group I mutations, which decreased the size of side chains, G164V and A183V increased the size of side chains. The hydrophobic core mutation reported previously, Multi-7, did not show any significant activity change even with a stability increase up to 9 kcal mol⁻¹ (28). It is also included in the plot for comparison, and the relationship between stability increase and activity change is also indicated (Fig. 4, blue square; dashed line).

Structural Basis of Metastability

In the crystal structure of the native α1AT (12), the stabilizing mutation sites are composed of many unusual interactions, which appear to be the basis of the metastability. These interactions are likely to be mobilized during the complex formation with a target protease. From the mutational analysis, the following modes were revealed as structural basis of the native metastability of α1AT.

Overpacking of Side Chains—Lys-335 interacts with hydrophobic residues, Ile-169 and Leu-172 (Fig. 5A). The Nζ atom of Lys-335 side chain may be paired with the backbone carbonyl oxygen of Ile-188 (2.87 Å) and Oy atom of Thr-165 (3.72 Å), but the aliphatic part of Lys-335 is squeezed in hydrophobic interactions provided by Ile-169 (3.45 Å) and Leu-172 (3.24 Å). It appears that K335G or K335A mutation relieved such strain by decreasing the size of the side chain. Remarkably, many stabilizing substitutions such as T165S, I169V, L172V, and L172A are the ones occurring at the residues interacting with Lys-335 (Fig. 5A). Furthermore, the substitution pattern showed size reduction as a common theme. Leu-172 is unusually close to Lys-335 (3.24 Å). L172V as well as L172A, which increased the stability even more (Table I), might have eliminated such an overpacking of the side chains. Stabilizing mutations such as F189I and F189V also showed the size reduction pattern. Size reduction to alanine at 189, however, resulted in the loss of stability. We previously reported that decrease in the size of side chains at the hydrophobic core of α1AT (Fig. 1, blue) confers increased stability (24). Structural determination of α1AT variants carrying stabilizing substitutions at the hydrophobic core revealed better packing at the mutation sites (12, 13, 31). The substitution pattern observed in the present study (Table I) revealed that size reduction in the present target region (Fig. 1, purple) also increases the stability of the molecule. It appears that overpacking of the side chains is one mechanism of the native strain of α1AT.

Polar-Nonpolar Interactions—The present studies revealed various polar-nonpolar interactions as an additional mode of destabilization in the target region. The side chain of Lys-335 is not only squeezed but also surrounded by hydrophobic residues. It is unusual to find charged residues in the hydrophobic environment. Studies on other mutant proteins revealed that the energetic cost of burying a charge in a hydrophobic environment is estimated be 3–9 kcal mol⁻¹ (32). Mutations at Lys-335 of α1AT increased the stabilizing energy by 3–7 kcal mol⁻¹ (Table I). Phe-189 is on a surface pocket (Fig. 5A), interacting unfavorably with the backbone carbonyl oxygen of Gly-164 (2.89 Å). Thr-165 appears to stabilize the end of helix F and the following turn by providing hydrogen bonding to the backbone carbonyl oxygen of Val-161 and backbone nitrogen of Ile-169. The Cy2 atom of Thr-165, however, has close interactions with the polar atoms of the backbone, while the hydroxyl

FIG. 5. A ribbon diagram of α1AT showing structural difference in the target region between the native (A) (12) and the cleaved (B) (14) structure of α1AT. The central β-sheet, A sheet, is shown at left, and the helix F (hF) and the following loop are shown on the right side. The side chains of representative polar residues are colored yellow. The atoms with a positive and a negative polarity are indicated in blue and red, respectively. The side chains of representative non-polar residues are shown in cyan. This figure was prepared using QUANTA program (Molecular Simulation Inc.).
group of Thr-165 is close to the Cε atom of Lys-335 (3.47 Å). T165S might have relieved such strain while maintaining the H-bond networks with backbone atoms of Val-161 and Ile-169. As expected, substitution of Thr-165 by alanine decreased stability. Leu-172 also has an unfavorable interaction with the backbone carbonyl oxygen of Asn-186 on s3A (2.97 Å). Lys-331 is on a surface pocket, interacting with Val-333 and Val-173. K331M increased the stability (Table I) possibly by providing more favorable interactions with the surrounding residues. It is likely that these unfavorable polar-nonpolar interactions are another basis of the native metastability of α1AT.

Cavities—Structural examination of the target region also revealed the existence of surface cavities. Two of the substitutions identified in the present study, A183V and G164V, do not fit the common theme of size reduction for stabilization of the native α1AT. In both cases, size increase rather than size reduction caused the stability increase. In the native structure (12), the region near Gly-164 and Ala-183 is not well packed, leaving an empty patch on the surface (Fig. 6). The surface cavity near Gly-164 is surrounded by the side chain atoms of Phe-189 and Lys-335. Likewise, the side chain of Ala-183 does not show much interaction with other side chains. Surrounding hydrophobic residues such as Phe-147, Ile-157, Val-185, and Leu-172 are at least 5 Å apart. Cavities are very likely to be the source of energetic cost in conformational stability (33). The stabilizing substitutions, G164V and A183V, appear to provide better packing by filling the nearby cavities.

Favorable Interactions in the Complex Form

In the cleaved relaxed form, the identified mutation sites have favorable interactions. The crystal structure of the serpin-protease complex is not available yet. However, it has been proposed that accompanying the structural transition to the relaxed complex form, the reactive center loop is at least halfway (up to P9 position, the 9th residue amino-terminal to the scissile peptide bond) inserted into A sheet (34–36), as in the relaxed cleaved structure. If this is the case, one can refer to the cleaved structure (14) to understand the complex. The side chain of Lys-335, which is buried in the native state (Fig. 5A), is exposed in the cleaved structure and makes a salt bridge with Asp-171 (Fig. 5B). Likewise, Lys-168 is paired with Glu-346 (P13 position) of the inserted reactive center loop (Fig. 5D). The side chain of Ile-169, which is exposed on the surface in the native form (Fig. 5A), interacts in the cleaved form with the side chain of Val-337 of s3A (Fig. 5D) and Ca atom of Gly-349 (P10 position) of the inserted reactive center loop. Residues at other mutation sites also are engaged in favorable interactions in the cleaved structure. It is very likely that the conversion from the native unfavorable interactions to more favorable interactions in the complex is the driving force for the conformational switch needed for the complex formation.

Mutations at Lys-168 and Ile-169, which decreased the inhibitory activity without increasing the stability (Fig. 4, triangles) suggest that the salt bridge between Lys-168 and Glu-346 shown in the cleaved structure (Fig. 5B) plays an important role in the complex stability. Likewise, Ile-169 appears to play a role in stabilizing the loop-inserted structure. It is not clear, however, if the salt bridge between Lys-335 and Asp-171 also plays a significant role in the complex formation, because D171N mutation did not affect the inhibitory activity significantly. It may be that Lys-335 mainly contributes to destabilization of the native form during the complex formation. It appears that each identified residue in the present target region contribute distinctively to the complex formation; some play a critical role in destabilizing the native form, some in the complex stability, and others in serving the dual role of destabilizing the native structure and stabilizing the complex. Many of these residues are conserved among inhibitory serpin sequences (2). The crystal structure of a serpin-protease complex will confirm the contribution of individual residues.

Importance of Local Strain in the Inhibitory Function

The mutational effect on the inhibitory activity observed in the present study is in contrast to that obtained previously with the stabilizing mutations at the hydrophobic core (Fig. 1, blue). The inhibitory activity of α1AT is very sensitive to the stabilization in the target region (Table I; Fig. 4). In contrast, the increase in the stabilization energy up to 9 kcal mol⁻¹ in the hydrophobic core by combining seven stabilizing single amino acid substitutions (F51L, T59A, T68A, A70G, M374I, S381A, and K387R; the mutant was named Multi-7) did not affect the inhibitory activity of α1AT toward target elastases (28). The results imply that local stability of the serpin is critical to inhibitory activity. It may be that some parts of the serpin molecule, especially the region where the reactive center loop is inserted, have to be loosened during the complex formation, whereas other parts like the hydrophobic core need not change as much. In this regard, it is interesting that while side chain locking among nonpolar side chains is the major cause of metastability in the hydrophobic core of α1AT (24), at the loop insertion site additional schemes like unfavorable polar-nonpolar interactions are observed. The polar groups may govern structural specificity during the complex formation. Internal polar groups are often found to be destabilizing (37, 38), but have evolved to impart structural uniqueness (37, 39). It appears that the local strain of inhibitory serpins is critical for relating the loaded energy to functional regulation.

2 H. Im and M.-H. Yu, unpublished results.
Implications for the Inhibitory Mechanism

Results in the present study strongly suggest that nature designed the native form of inhibitory serpins to be poorly folded with the purpose of carrying out a sophisticated regulation of protease inhibition. Our study also revealed that various folding defects such as side chain locking, buried polar groups in unfavorable hydrophobic environments, and cavities are the structural basis of the metastability of $\alpha_1$AT. These folding defects appear to be designed to destabilize the interaction of helix F and the following loop with A sheet (Fig. 1). Especially, Lys-335 is in a strategic position for opening A sheet; the side chain of Lys-335 in the uncleaved native form is tightly squeezed by the surrounding hydrophobic residues from helix F and the following loop that covers A sheet (Fig. 5A). Considering the location (Fig. 1) and energetic cost (Fig. 2; Table I) of Lys-335, we propose that this lysine plays a pivotal role during the complex formation. This lysine is squeezed by the surrounding hydrophobic residues from helix F, resulting in the execution rather than a consequence of functional reconciliation rather than a consequence of functional reconciliation.

Interestingly, most of the substituting residues in stable suboptimal $\alpha_1$AT are the ones already existing in the sequence of ovalbumin (Table I), a non-inhibitory member of the serpin family. Ovalbumin and inhibitory serpins share a common ancestor (47). The molecule might have evolved for better folding and stability in the ovalbumin line, but for acquiring inhibitory function in the inhibitory serpin line.

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