단백질의 구조 전환과 기능 조절

Conformational Switch and Functional Regulation of Proteins

유명희
(Myeong-Hee Yu.)

Abstract

In common globular proteins, the native form is in its most stable state. However, the native form of inhibitory serpins (serine protease inhibitors) and some viral membrane fusion proteins is in a metastable state. Metastability in these proteins is critical to their biological functions. Our previous studies revealed that unusual interactions, such as side-chain overpacking, buried polar groups, surface hydrophobic pockets, and internal cavities are the structural basis of the native metastability. To understand the mechanism by which these structural defects regulate protein functions, cavity-filling mutations of α1-antitrypsin, a prototype serpin, were characterized. Increasing conformational stability is correlated with decreasing inhibitory activity. Moreover, the activity loss appears to correlate with the decrease in the rate of the conformational switch during complex formation with a target protease. We also increased the stability of α1-antitrypsin greatly via combining various stabilizing single amino acid substitutions that were distributed throughout the molecule. The results showed that a substantial increase of stability, over 13 kcal/mol, affected the inhibitory activity with a correlation of 11% activity loss per kcal/mol. The results strongly suggest that the native metastability of proteins is indeed a structural design that regulates protein functions and that the native strain of α1-antitrypsin distributed throughout the molecule regulates the inhibitory function in a concerted manner.

Key Words : protein, conformation, stability, functional regulation, structural design

1. 서 론[1]

The native forms of most proteins are thermodynamically the most stable state (1). However, the native forms of some proteins are metastable: typical example are the strained native structure of plasma serpins (serine protease inhibitors) (2) and the membrane fusion proteins of some virus (3). The native strain of serpins is crucial to their physiological function (2,4). The serpins include α1-antitrypsin (AT), α1-antichymotrypsin, C1-inhibitor, plasminogen activator inhibitor-1, antithrombin-III, and antiplasmin, which regulate processes such as inflammation, coagulation, fibrinolysis, and complement activation (2,4). The serpin structure is composed of three β-sheets and several α-helices, and the reactive center loop is exposed at one end of the molecule for protease binding (Fig. 1). Upon binding a target protease, the reactive center loop of serpins is inserted into

한국과학기술연구원
(서울시 성북구 하월곡동 39-1,
Fax: 02-958-6919
E-mail : mhyu@kist.re.kr)
Some of this work was published in *Proc. Natl. Acad. Sci. USA*, vol. 97, 7727 (2000).

the major β-sheet, A sheet, to form a very stable complex between the inhibitor and the protease (5). Since the metastable native form has an advantage of facile conversion into an alternative more stable conformation, it is conceivable that the native metastability of serpins is utilized for the facile conformational change during the complex formation.

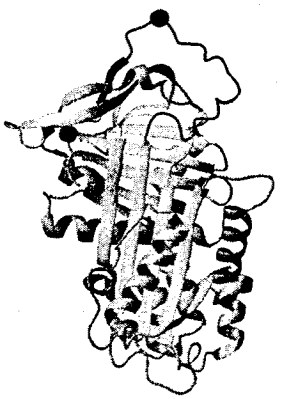

Fig. 1. Schematic drawing of AT structure.

To understand the structural basis and functional role of the native metatsability, we have characterized stabilizing amino acid substitutions of AT, a prototype inhibitory serpin (6-10). Mutational patterns and structural examination of the mutation sites revealed various unusual interactions, such as overpacking of the side-chains in the interior of protein, buried polar groups, surface hydrophobic pockets, and internal cavities as the structural basis of native metastability in AT (6-10). Cavities and unfavorable polar-nonpolar interactions were also found in the crystal structures of both mature and precursor forms of hemagglutinin, the membrane fusion protein of influenza virus (11,12). These structural defects are likely to be the design principles of the metastable native proteins to regulate their functions. However, the mechanism of how these structural defects regulate the protein functions has yet to be elucidated. In the present study, we characterized cavity-filling mutations of AT that increased the conformational stability. The results show that the native metastability regulates the inhibitory function of AT by controlling the rate of the conformational switch during complex formation with a target protease. We also examined very stable mutations made by combining various single residue substitutions that were distributed throughout the molecule. Characterization of the stable mutations suggests that the strain of AT scattered over the molecule does regulate the inhibitory activity in a concerted manner.

2. 실 험

Materials. The plasmids for AT expression in *Escherichia coli* and the preparation of recombinant proteins were described previously (6-8). Mutations were introduced by oligonucleotide-directed mutagenesis.

Equilibrium unfolding of AT variants. Equilibrium unfolding was monitored by spectrofluorimetry (λex = 280 nm, λem = 360 nm) at 25 ℃ in a buffer (10 mM phosphate, pH 6.5/ 50 mM NaCl/ 1 mM EDTA) containing various concentrations of GdmCl. Equilibrium unfolding was fitted to a two-state model as described previously (6-8).

Inhibitory parameters of AT variants. The stoichiometry of inhibition (SI) towards porcine pancreatic elastase (PPE) was measured (13). Various amounts of AT variants were incubated with 100 nM PPE in 30 mM phosphate, 160 mM NaCl, 0.1% PEG 6000, and 0.1% Triton X-100. After incubation at 37 ℃ for 10 min, the reaction mixture was diluted 10-fold with the same buffer and the residual protease activity was determined using *N*-succinyl-$(Ala)_3$-*p*-nitoranilide as a substrate. Linear regression analysis of the decrease in proteinase activity with increasing molar ratio of AT over PPE yielded the estimate for the SI as the intercept on the abscissa.

Stopped-flow measurement of conformational switch. Time-resolved fluorescence change was measured using SFM-4 stopped-flow apparatus (Bio-Logic). Experiments were carried out in 10

mM phsphate (pH 6.5), 50 mM NaCl, and 1 mM EDTA at 25 ℃. For the reaction of NBD (*N,N*-dimethyl-*N*-(acetyl)-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine)-labeled AT and PPE, NBD probe was attached to P9 position of the reactive center loop. Reactions were carried out at a final concentration of 60 nM NBD-AT and at various concentrations of PPE. The fluorescence of NBD was excited at 480 nm, and the change in fluorescence above 495 nm was measured using a filter with 495 nm cut-off.

3. 결과 및 고찰

Effect of cavity-filling mutations. In the native structure of AT, Gly 117 is surrounded by Phe 119, Tyr 160, and Val 185 on the outer face of β-sheet A. The site, initially identified by a thermostable mutation (G117V: substitution of glycine 117 by valine) in screening, appears to form a cavity, having the nearest side-chain carbon atoms 4.8 Å apart. Cavities are a source of energetic cost in protein stability (14). Gly 117 residue was replaced with a series of other hydrophobic amino acids. The conformational stability and activity of AT variants were measured. Fig. 2 shows that activity decrease reflected in the increase of SI values correlates strongly with increases in the stability of AT. These results clearly establish an inverse correlation between the conformational stability and the inhibitory activity of AT: an increase in the conformational stability is detrimental to the protein function.

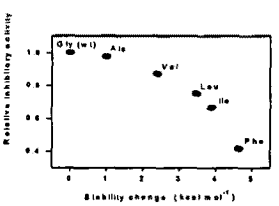

Fig. 2. Inverse correlation between the stability and the activity of AT variants.

Retardation of conformational switch by stability increase. Complex formation of a serpin with its target protease accompanies a massive conformational change in the serpin molecules: the reactive center loop is inserted into β-sheet A as the 4th strand while the protease is covalently linked to the reactive center loop as an acyl intermediate (5). In order to probe the loop insertion process during the complex formation, we introduced cysteinyl residue at the P9 position of the reactive center loop (Fig. 1), and labeled it with a fluorescent probe, NBD. The NBD-labeling did not alter the inhibitory activity significantly. We measured how fast the probe moves into a hydrophobic environment by measuring the rate of NBD fluorescence increase when AT binds to PPE. The change of NBD fluorescence was measured under pseudo first order reaction condition by rapid mixing of NBD-labeled AT with increasing amount of PPE using stopped-flow fluorometry. The observed rate constants (*kobs*) increased with increasing PPE concentrations, and the limiting rate constants of NBD fluorescence change were obtained by fitting the plot of *kobs* vs. [PPE] to a hyperbolic equation. Table 1 shows that the rate of NBD fluorescence change decreased gradually as the protein stability increased. The results indicate that increasing the conformational stability of AT retards the docking of the P9 position of the reactive center loop into the main body of the molecule.

Table 1. Inhibitory activity and conformational switch rate of AT variants.

Residue	SI	k_{obs} (s^{-1})
Gly Iwt)	1.66±0.66	2.05
Ala	1.70±0.03	ND
Val	1.91±0.05	2.00
Leu	2.21±0.09	ND
Ile	2.50±0.08	1.93
Phe	3.99±0.12	1.65

Relationship between global stability and inhibitory activity of AT. Among the stabilizing single residue substitutions of AT distributed at 50 residue sites throughout the molecule, those that affected the inhibitory activity due to increase in stability are localized in the regions where the reactive site loop is inserted during complex formation with a target protease (10). This raises the question on the role of the strain distributed over the whole molecule. To address this point, we increased the stability of AT greatly via combining various stabilizing single amino acid substitutions that did not affect the activity individually. Gradual loss of inhibitory activity was observed as the stability increased over 13 kcal/mol. The correlation between the stability increase of AT and the decrease in the inhibitory activity was about 11% activity loss per kcal/mol. The results suggest that the native strain of AT distributed throughout the molecule regulates the inhibitory function in a concerted manner.

4. 결 론

Our results strongly suggest that the native form of serpins has evolved as a poorly folded structure that enables sophisticated regulation of protease inhibition. Perhaps the conformational switch is driven by mobilization of unfavorable interactions in the native state into more favorable ones. In conclusion, the native metastability of proteins, seemingly due to folding defects, is indeed a structural design that regulates protein functions presumably by facilitating conformational switch. The strain distributed throughout the molecule regulates the function in a concerted manner.

감사의 글

This research was supported by National Creative Research Initiatives grant from the Korean Ministry of Science and Technology.

참고 문헌

[1] Anfinsen, C. B. *Science*, **181**, 223-230 (1973)

[2] Huber, R. & Carrell, R. W. *Biochemistry* **28**, 8951-8966 (1989)

[3] Carr , C. M. & Kim, P. S. *Cell* **73**, 823-832 (1993)

[4] Stein, P. E. & Carrell, R. W. *Nature Struct. Biol.* **2**, 96-113 (1995)

[5] Huntington, J. A., Read, R. J., and Carrell, R. W. *Nature* **407**, 923-926 (2000)

[6] Lee, K. N., Park, S. D., & Yu, M.-H. *Nature Struct. Biol.* **3**, 497-500 (1996)

[7] Lee, K. N., Im, H., Kang, S. W., & Yu, M.-H. *J. Biol. Chem.* **273**, 2509-2516 (1998)

[8] Im, H., Seo, E. J., & Yu, M.-H. *J. Biol. Chem.* **274**, 11072-11077 (1999)

[9] Ryu, S.-E., Choi, H.-J., Kwon, K.-S., Lee, K. N., & Yu, M.-H. *Structure* **4**, 1181-1192 (1996)

[10] Seo, E. J., Im, H., Maeng J.-S., Kim, K. E., and Yu, M.-H. *J. Biol. Chem.* **275**, 16904-16909 (2000)

[11] Bullough, P. A., Hughson, F. M., Skehel, J. J., & Wiley, D. C. *Nature* **371**, 37-43 (1994)

[12] Chen, J, Lee, K. H., Steinhauer, D. A., Stevens, D. J., Skehel, J. J., & Wiley, D. C. *Cell* **95**, 409-417 (1998)

[13] Patston, P. A., Gettins, P., Beechem, J., & Schapira, M. *Biochemistry* **30**, 8876-8882 (1991)

[14] Eriksson, A. E., Baase, W. A., Zhang, X. J., Heinz, D. W., Blaber, M., Baldwin, E. P., & Matthews, B. W. *Science* **255**, 178-183 (1992)