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The concept of the unfolding region for approaching the mechanisms of enzyme stabilization

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Abstract

Starting from the hypothesis that chemical or genetic modifications as methods of protein stabilization are most successful if the most labile region of the molecule is strengthened, the importance of local structural regions and their modification for thermal stability has been investigated on two enzyme systems. The thermal unfolding of ribonuclease A and its glycosylated form, ribonuclease B, as well as of partially deglycosylated derivatives of ribonuclease B was studied by limited proteolysis. From the analysis of the primary proteolytic cleavage sites and the kinetic stabilities, it can be concluded that the unfolding pathway of ribonuclease A is not changed by the carbohydrate attachment and that the higher stability of ribonuclease B results from the first *N*-acetylglucosamine residue. On neutral protease from *Bacillus stearothermophilus* where a surface-located region (residues 56 to 69) is known to be crucial for the thermal stability of the enzyme, the introduction of a disulfide bridge into this region by site-directed mutagenesis resulted in an extreme stabilization of the molecule. Cys residues, which were introduced into different positions of the protein surface, enabled the specific immobilization of the corresponding single mutant enzymes via their SH groups to Activated Thiol-Sepharose. The resulting stabilization effects were highest if the attachment of the enzyme to the carrier was within the residues 56 to 69.

Keywords: Enzyme stabilization; Unfolding region; Immobilization; Ribonuclease A; Ribonuclease B; Neutral protease; *Bacillus stearothermophilus*; Glycosylation; Limited proteolysis; Site directed mutagenesis; Thermal inactivation

1. Introduction

The resistance of the catalytically active protein structure toward higher temperatures and other denaturing influences is one of the most important criteria in the application of enzymes. Modifications of the protein structure by site directed mutagenesis or binding to polymeric carriers belong to the mostly applied approaches for enzyme stabilization [1–3]. The molecular basis of protein stabilization, however, is still poorly understood, and the success of most stabilizing methods has remained a matter of trial and error. Our previous studies on immobilized enzymes [4–6] resulted in a new concept of protein stabilization, where the success or failure of any stabilizing approach is related to the position of the carrier attachment in the protein molecule. In the present paper, recent studies for its verification and its extension to protein stabilization by other methods such as

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modification by glycosylation or site directed mutagenesis are summarized.

2. The model of the unfolding region

The covalent and also the adsorptive binding of enzymes to polymers is often, but not always, connected with an increase of stability. We studied the thermal inactivation of monomeric enzymes such as α-amylase, chymotrypsin, or trypsin before and after immobilization to different silica, polystyrene, or polyacrylamide matrices [4–6]. In all cases the soluble enzymes were irreversibly inactivated according to a first-order kinetics, whereas the immobilized enzymes, if stabilized, showed a distinct biphasic inactivation progress. The inactivation kinetics of the biphasic inactivation curves could be excellently fitted by the sum of two exponential terms, where one rate constant was in the range of the inactivation constant of the soluble enzyme whereas the second one was markedly smaller. Experiments including active site titration on immobilized trypsin and the determination of K_M values and Arrhenius activation energies for immobilized α-amylase and chymotrypsin at different stages of thermal inactivation indicated that the biphasic inactivation kinetics are caused by two enzyme species arising as a consequence of immobilization, which differ in stability but not in the catalytic properties. The kinetic studies were supplemented by following the unfolding process by ESR and fluorescence measurements [6,7]. The existence of two discrete enzyme subpopulations resulting from carrier binding and differing in their unfolding behaviour was indicated.

Together with other findings and observations, these results led us to a model which might be a key for the understanding of enzyme stabilization by protein modification (Fig. 1). It is based on the conception that unfolding of an enzyme molecule under denaturing stress starts at a definite region of the molecule, denoted as unfolding region. After immobilization the unfolding region of the molecule may be either free or fixed by covalent or noncovalent bonds. All molecules, in which the unfolding region is free, are unfolded in the same way and with the same rate constant as the soluble enzyme. These molecules represent the labile fraction. In the other enzyme molecules, forming the stable fraction, the unfolding region is strengthened. As consequence, the normal unfolding pathway is energetically blocked, and the loss of activity is the result of a different unfolding pathway. According to this model, stabilization of a protein by immobilization or by other modifications is dependent on the position of the modification and will be most successful when the most labile structural region of the surface of the molecule is strengthened.

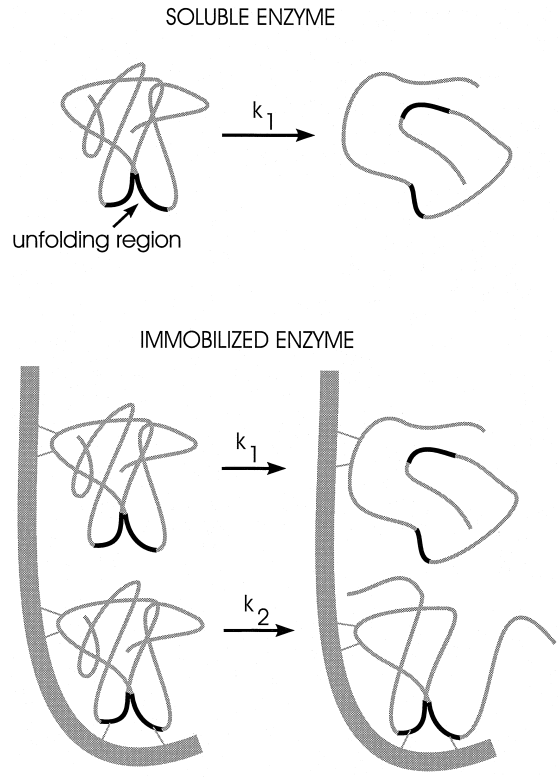

Fig. 1. Model of the unfolding region. Unfolding of the soluble protein molecule is initiated at its most labile site. Immobilization results in stabilization if this unfolding region is fixed, whereas no stabilization is observed if the unfolding region is free.

3. Localization and modification of the unfolding region in bovine pancreatic ribonuclease

On ribonuclease A, a small protein molecule of well-known structure (Fig. 2) and stability behaviour, the proteolytic degradation was studied with the aim to localize the unfolding region of this molecule [8]. Trypsin and thermolysin proved to be appropriate for this purpose, because these proteases do not attack the native molecule but degrade the protein as soon as it is unfolding. The primary cleavage sites of ribonuclease A could be identified by partial N-terminal sequencing of the fragments after blotting of the electrophoretic bands in combination with matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) after separation of the proteolytic fragments by HPLC (Table 1). They were determined to be Lys31–Ser32 and Arg33–Asn34 for trypsin and Asn34–Leu35 and Thr45–Phe46 for thermolysin. Hence, the structural region from Lys31 to Leu35, together with the adjacent β-structure containing Thr45–Phe46, is suggested to represent a labile region in terms of the proposed unfolding region.

Interestingly, naturally occurring ribonuclease B, which has the same amino acid sequence and tertiary structure but is more stable than ribonuclease A, differs by an N-linked oligosaccharide chain in even that region, at

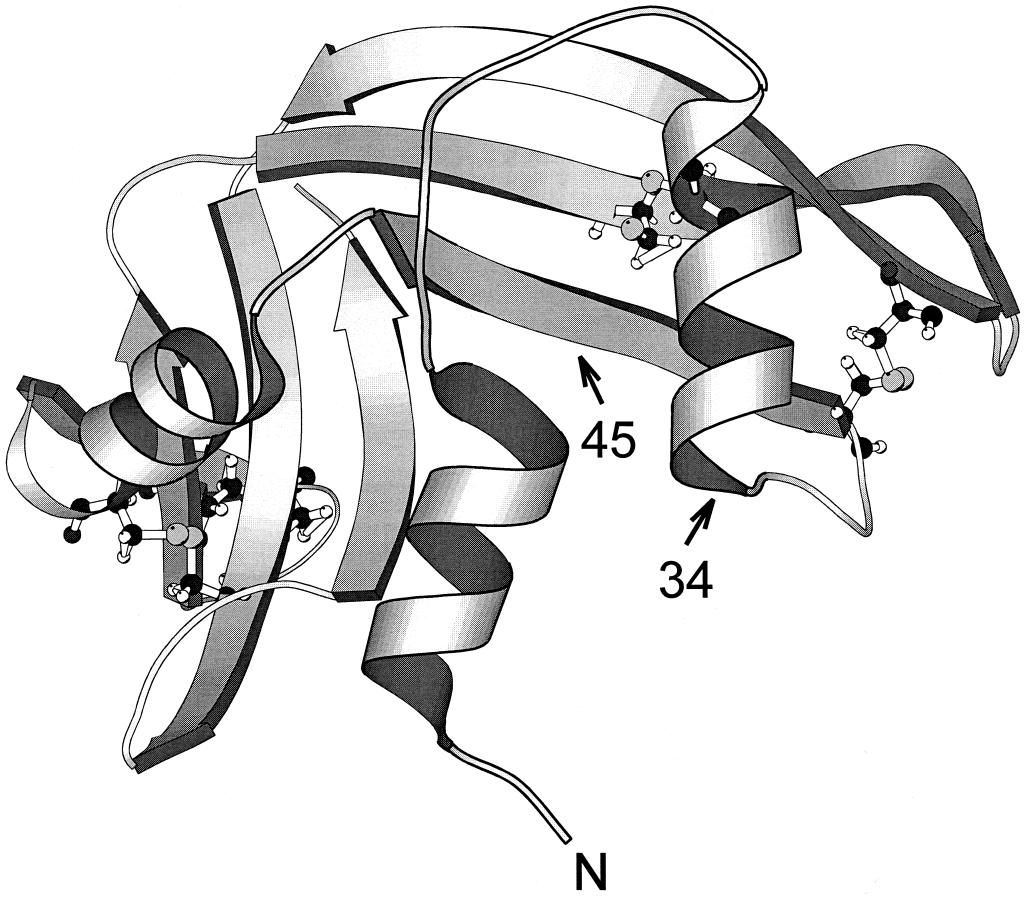

Fig. 2. Tertiary structure of ribonuclease A. The model was taken from the Brookhaven data bank and drawn with MOLSCRIPT, version 1.4. Ribonuclease A contains 124 amino acid residues and four disulfide bridges. The primary cleavage sites for thermolysin are indicated by arrows.

Table 1
Complementary fragments of ribonuclease A and ribonuclease B obtained by limited proteolysis with thermolysin at 65°C

Fragment	Ribonuclease A		Ribonuclease B	
	Molecular mass (Da)	Sequence	Molecular mass (Da)	Sequence
I	3857	1–34	5071[a]	1–34
II	5172	1–45	6386[a]	1–45
III	9003	46–124	8996	46–124
IV	10 313	35–124	10 310	35–124

Molecular masses were determined by MALDI-MS after separation of the fragments by HPLC. Sequences were assigned by N-terminal sequencing. Experimental details are given in Refs. [8,10].
[a]Lowest molecular mass in mannose series.

Asn34. A comparison of the kinetic and thermodynamic thermal stabilities of ribonuclease A and ribonuclease B revealed slightly higher stabilities of ribonuclease B ($\Delta\Delta G^{\#} = 2.2 \pm 0.3$ kJ mol^{-1} and $\Delta\Delta G = 2.5 \pm 0.2$ kJ mol^{-1} at 60.4°C, the transition temperature of ribonuclease A) due to the carbohydrate moiety [9]. The analysis of the unfolding process of ribonuclease B by limited proteolysis (Table 1), however, indicated again the structural region in the vicinity of Lys31 and Leu35 as being unfolded first [10]. Obviously, the slight stabilization of ribonuclease B compared to ribonuclease A is not connected with a significant change of the unfolding pathway.

In order to analyze the effect of glycosylation at the unfolding region on the stability of ribonuclease, we continued this series of experiments by modifying the carbohydrate moiety. On one hand, the carbohydrate chain was shortened by glycosidases and, on the other hand, it was extended by the specific binding to Concanavalin A or to Con A-Agarose (Fig. 3). The partial deglycosylation of ribonuclease B, which contains two *N*-acetylglucosamine residues and 5–9 mannose residues, was performed by endoglycosidase F or α-mannosidase and resulted in homogeneous products after HPLC separation, which were identified by MALDI-MS [11]. The deglycosylation left only one *N*-acetylglucosamine residue with endoglycosidase F, while both *N*-acetylglucosamine residues and three mannose residues remained after deglycosylation with α-mannosidase. As a result six ribonuclease species with differently large moieties in the unfolding region (Fig. 3) were available to be compared with respect to their kinetic stability towards thermal denaturation. Fig. 4

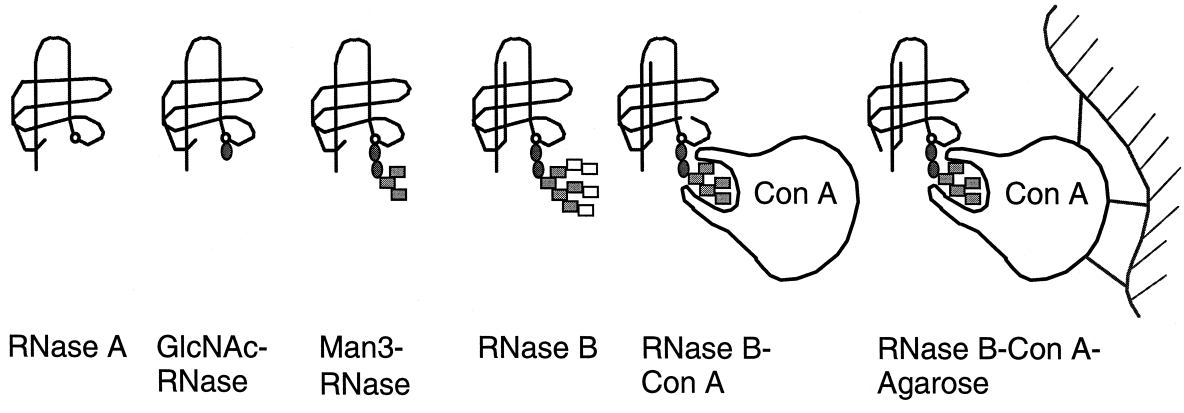

Fig. 3. Schematic presentation of the naturally occurring and modified ribonuclease species. ○ Asn34, ● GlcNAc, ■ Man (1–5), □ Man (6–9).

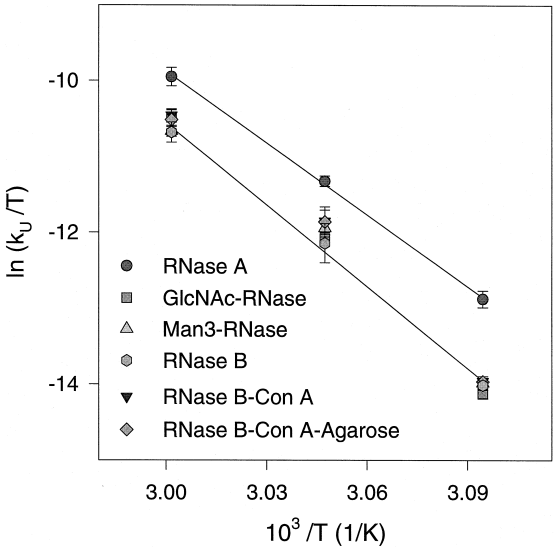

Fig. 4. Eyring plot of the thermal unfolding constants of the naturally occurring and modified ribonuclease species. The thermal unfolding constants k_u were determined by a method combining limited proteolysis and quantitative sodium dodecylsulfate polyacrylamide-gel electrophoresis [9,11].

shows the Eyring plots of the thermal unfolding constants demonstrating that all carbohydrate-containing ribonuclease species possess the same stability within the experimental error, which is, however, significantly higher than that of the nonglycosylated ribonuclease A. Even the immobilization to Con A-Agarose does not further increase thermal stability. The results prove that the stability difference between ribonuclease A and ribonuclease B has to be attributed to the attachment of the first carbohydrate unit to Asn34.

4. Strengthening of the unfolding region of neutral protease (*B. stearothermophilus*) by site-directed mutagenesis and immobilization

Another model system to study the role of local surface regions of the protein molecule for

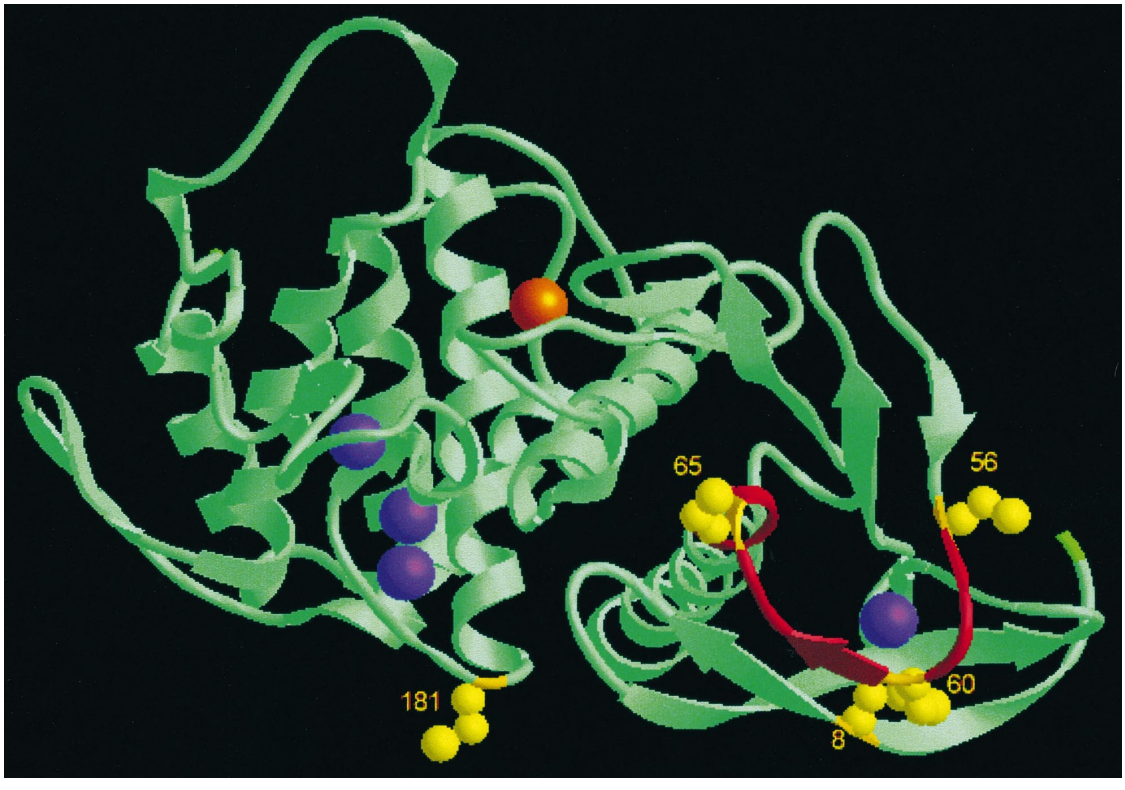

Fig. 5. Structural model of the neutral protease from *B. stearothermophilus*. The unfolding region 56–69 is drawn in red colour. The large spheres indicate the Zn^{2+} ion (orange) in the active site and the four Ca^{2+} ions (purple) bound to the molecule. Yellow indicates the positions where Cys residues were introduced into the enzyme. In the immobilization studies each mutant contained only one Cys.

its stability is represented by mutant enzymes of a bacterial neutral protease of the thermolysin type. Studies on the relation between amino acid exchanges and thermal stability by Eijsink et al. [12] had shown that a surface-located region in the N-terminal domain of this enzyme (residues 56 to 69) is crucial for the thermal stability of the enzyme, whereas other regions are less significant (Fig. 5).

On this system, we studied stabilization effects by two approaches. Based on computer-aided protein design, a double mutant was constructed in which two cysteine residues were introduced into the positions 60 (within the sensitive region) and the neighboured position 8 instead of Gly and Asn, respectively. These substitutions resulted in the spontaneous formation of a disulfide bridge and an extreme stabilization of the molecule [13]. The enzyme had a half-life of more than 30 min even at 92.5°C, whereas the wild type enzyme showed a comparable half-life at 75°C. In contrast, the single mutant enzymes were less stable than the wild type enzyme.

The second study on this model system started from mutant enzymes, in which cysteines were introduced into different positions of the enzyme surface by site directed mutagenesis (Fig. 5) with the aim to immobilize the enzyme specifically at different positions of the surface, within the unfolding region and remote from it, via one-point bindings and to probe the dependence of stabilization effects on the position of the carrier binding. The mutant enzymes showed specific activities comparable to that of the wild-type. Specific immobilization of the Cys-mutant enzymes was obtained with Activated Thiol-Sepharose (Fig. 6), where activities, related to those of the soluble enzymes were between 50 and 100%. In all cases thermal inactivation followed irreversible first-order kinetics with remarkable differences of the half-lives in dependence of the position of the immobilization site (Table 2). Binding of the enzyme via engineered cysteines in the critical unfolding region (in the positions 56, 60 and 65) resulted in stronger stabilization than in other regions (postion 8 or position 181). An extremely strong

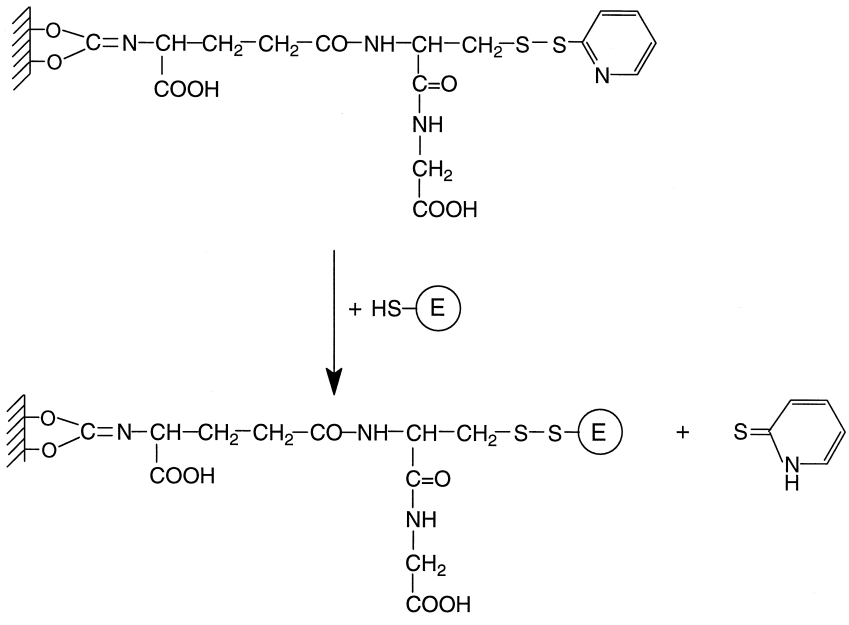

Fig. 6. Enzyme binding to Activated Thiol-Sepharose. SH-containing mutant enzymes react with Activated Thiol-Sepharose (Pharmacia) via disulfide exchange. Experimental details are given in Ref. [14].

Table 2
The influence of directed immobilization on the thermal stability of mutant neutral protease from *B. stearothermophilus*

Mutant enzyme	$\tau_{1/2\,(\text{immobilized})} / \tau_{1/2\,(\text{soluble})}$
G8C	1.3 ± 0.1
T56C	23.7 ± 0.6
N60C	5.1 ± 0.2
S65C	9.6 ± 0.4
N181C	3.3 ± 0.1

The mutant enzymes, soluble and immobilized to Activated Thiol-Sepharose, were incubated at 75°C and assayed after fast cooling. Half-lives of the soluble ($\tau_{1/2\,(\text{soluble})}$, min^{-1}) and immobilized ($\tau_{1/2\,(\text{immobilized})}$, min^{-1}) enzymes were determined from the inactivation progress that followed first-order kinetics. Experimental details are given in Ref. [14].

stabilization was observed with the mutant engineered in position 56, demonstrating the great significance of the position of protein modification for enzyme stabilization.

5. Conclusions

In summary, our results and experiences support the idea that the success of a method for protein stabilization strongly depends on the position where the molecule is modified. Therefore, a directed design of modification for the stabilization can be only reached with knowledge of the individual structure of the protein molecule and additionally with knowledge of its most labile structural regions. Since these properties are not yet known for most enzymes, their stabilization by chemical modification such as immobilization will remain a more or less empiric matter.

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