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Effects of chemical modification of lysine residues in trypsin

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Abstract

Chemical modifications are a simple method to identify and modify functional determinants of enzymes. In the case of serine proteases, it is possible to induce characteristics which are advantageous for peptide synthesis. In this work, we investigated the influence of guanylation and succinylation of lysine residues on the S′-subsite specificity, the catalytic behavior and stability of trypsin. We have found, that succinylation leads to an about 10-fold better acceptance of basic residues in P1′, whereas guanylation shows no remarkable effects. Furthermore, guanylation enhances, succinylation reduces the general enzyme–substrate interactions in P2′. The structural fundamentals of these specificity changes are discussed. The catalytic behavior of trypsin was not influenced by guanylation and succinylation but an enhancement of the stability against autolytic processes by introducing additional negative charges into the protein was observed.

Keywords: Peptide synthesis; Protease; Acyl transfer; Enzyme specificity; Enzyme modification

1. Introduction

The understanding of the structural basis of enzyme specificity is a major goal of enzymology. The trypsin family of serine proteases is an ideal model system to address this problem. Thus, several groups performed protein engineering in order to identify the structural determinants of trypsin specificity. These studies contribute to the development of specific protease inhibitors, the design of site specific proteases and the utilization of proteases as catalysts in enzymatic peptide synthesis. In order to increase stability or alter activity and specificity of enzymes, the application of chemically or genetically modified proteases has become very attractive.

Genetic methods are useful tools for identifying and altering the specificity of enzymes [1–3] and modulate their catalytic behavior thereby uncovering detailed insights into the catalytic mechanism and the structural basis of enzymatic activity [4,5]. Using genetic engineering, it is also possible to influence the stability of the

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enzyme [6]. In contrast to genetic methods, chemical modification is a generally applicable tool because modified biomolecules are easy to prepare in a large scale from commercial material. Furthermore, the behavior of biomolecules with non-proteinogenic amino acids can be studied. Chemical modifications are basic principles in the nature to alter characteristics of biomolecules. The adaptation of this method to bioorganic chemistry leads to a variety of biomolecules which cannot be generated by genetic engineering. As well as genetic methods, chemical modification may modulate the specificity [7], activity [8] and stability of enzymes [9]. In the case of trypsin, lysine residues are considered as suitable targets for chemical modification aimed at the modulation of these properties. For instance, the proteolysis of the Lys^{15}–Ile^{16} bond converts trypsinogen into fully active single chain β-trypsin. The cleavage between Lys^{145}–Ser^{146} of β-trypsin generates double chain α-trypsin [10]. Effects on enzyme activity result also from the cleavage of the Lys^{60}–Ser^{61} bond [11]. Lys^{90} and Lys^{107} are further autolytic cleavage sites. Lysine residues are not only incorporated in proteolytic regulation processes. They also contribute to the specificity of trypsin. For example, Lys^{188}, Lys^{222} and Lys^{224} are parts of two surface loops which are determinants of the primary speci-

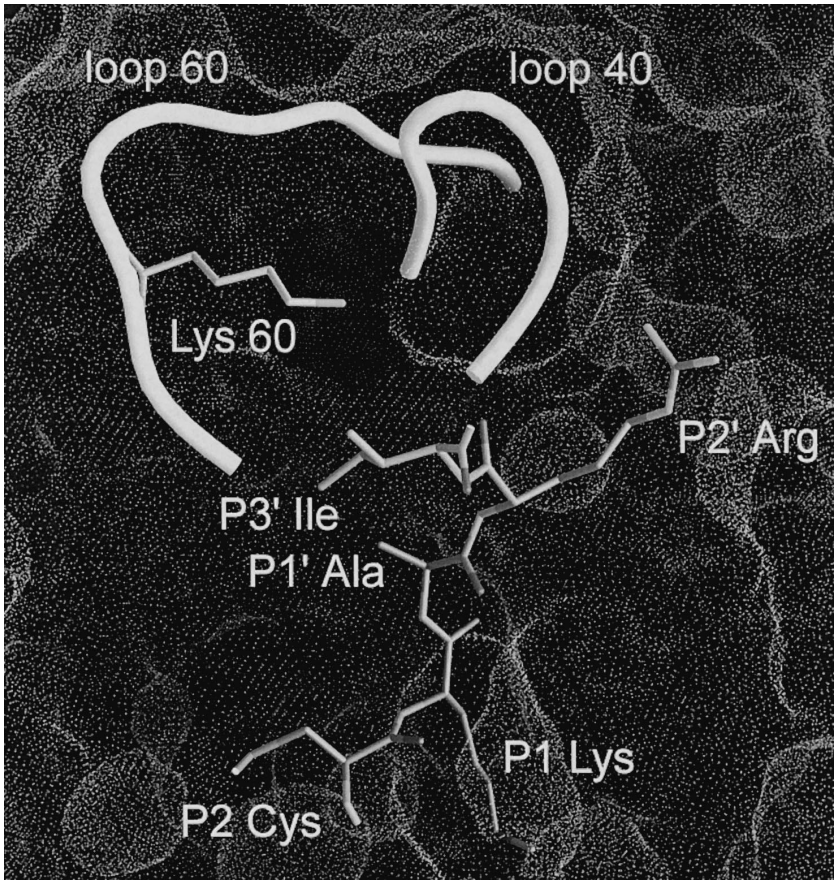

Fig. 1. S′ subsites of trypsin. The structure of the rat trypsin (G226D/D189S)-BPTI complex (PDB-Code 1brb) has been processed with Grasp. The position of the P2–P2′ residues of the BPTI inhibitor and the two loops (loop 60 from residue 57 to 64, loop 40 from residue 33 to 41) of trypsin are shown.

ficity of trypsin. The identification of these determinants was executed by the exchange of analogous segments of chymotrypsin aimed at the manifestation of chymotryptic specificity in trypsin. The reconstitution of chymotrypsin-like activity in trypsin required the replacement of two surface loops (loop 1 residues 185–188, loop 2 residues 221–225) by the analogous loops of chymotrypsin. Furthermore, Lys^{60} is a component of the S′-binding site in trypsin which side chain may interact directly with the P1′ amino acid residue (nomenclature according to Schechter and Berger [12]) in the substrate (Fig. 1). The mutation K60E resulted in a dramatic change of the S1′ specificity in trypsin [13]. The preference for basic P1′ amino acid residues was increased by one to two orders of magnitude. Molecular modeling studies revealed that a formation of a salt bridge between the carboxyl group of Glu^{60} and the guanidino/amino function of P1′ Arg/Lys in the substrate is responsible for the strong preference of basic residues in P1′ whereas in trypsin, only hydrogen bonds between the basic residue in P1′ with backbone carbonyl groups occur.

In this work, we compare the effects of chemical modification of lysine residues in trypsin by succinylation and guanylation. We describe the consequences of these modifications on the activity, specificity and stability of the enzyme.

2. Experimental

2.1. Chemicals

Amino acid derivatives were obtained from Bachem (Switzerland), 1-guanyl-3,5-dimethylpyrazole from Serva (Germany), 4-nitrophenyl-4-guanidinobenzoate-hydrochloride (pNPGB) from Merck (Germany), succinic anhydride from Sigma (Germany). Fluorogenic peptide substrates were synthesized using standard Fmoc solid phase protocols and used for enzyme kinetics as described by Grahn et al. [14].

2.2. Enzymes

Bovine trypsin was obtained from Serva (Germany). Guanidinated trypsin was produced using 1-guanyl-3,5-dimethylpyrazole with reference to Habeeb [15], succinated trypsin was produced using succinic anhydride according to Klotz [16] followed by dialysis against 1 mM HCl. Enzyme concentrations were determined by active site titration with pNPGB.

2.3. Acyl transfer

Acyl transfer reactions were carried out in 1.5 ml polypropylene tubes in total volumes of 50 μl at pH 8.0 (50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$) and 25°C. The final concentration of Bz-Arg-OEt was 2 mM and the nucleophile concentrations were 25 mM which were calculated as unprotonated amino acid amide and dipeptide concentration [N] according to Eq. (1) ($[N]^0$ corresponds to the total nucleophile concentration):

$$[N] = [N]^0/(1 + 10^{pK-pH}) \quad (1)$$

The pK values of the α-amino group of the nucleophiles were determined by inflection point titration on a Vidio-Titrator VIT 90 (Radiometer, Denmark). Acyl transfer reactions were initiated by the addition of enzyme leading to enzyme concentrations of 15–100 nM. The enzymatic reaction was stopped by addition of 50% aqueous methanol and 1% trifluoracetic acid. The composition of the reaction mixture was monitored by HPLC at 254 nm on a reversed phase C 18 column using 12–20% acetonitrile (254 nm, flow rate 1 ml/min) and determined when no secondary hydrolysis was detected. Since acyl donor, hydrolysis and aminolysis products contain the same chromophor, their molar extinction coefficients were assumed to be equal.

2.4. Autodigestion

To follow the autodigestion, the native enzyme and the modified forms were incubated at

37°C in 100 mM Tris–HCl buffer pH 8.0 in the presence of 20 mM $CaCl_2$ or 1 mM EDTA. The enzyme concentrations were 10^{-6} M. At incremental time intervals, autodigestion was stopped by addition of aliquots to 1 M HCl to a final pH of 3. Residual activity of each sample was measured using Bz-Arg-pNA as substrate and carried out in 20 mM Tris–HCl pH 8.0, 100 mM NaCl and 5 mM $CaCl_2$. Liberated pNA was determined photometrically at 410 nm.

3. Results and discussion

3.1. Hydrolysis kinetics

The general catalytic behavior of trypsin and the modified forms was examined by steady-state hydrolysis kinetic studies. As Table 1 indicates, the k_{cat}/K_M values for the hydrolysis of Bz-Arg-pNA are comparable. This result was verified by the use of longer peptide substrates which usually better reflect the enzyme–substrate interactions. Also in these cases, we observed only small differences up to one order of magnitude. Consequently, chemical modification of lysine residues by succinylation and guanylation does not affect the catalytic behavior.

Table 1
Kinetic parameters of bovine trypsin, guanyl-trypsin and succinyl-trypsin on amide substrates[a]

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\,s^{-1}$)
Bz-Arg-pNA[b]			
trypsin	2.36	3.80	1.61×10^3
guanyl-trypsin	4.77	3.81	7.99×10^2
succinyl-trypsin	1.00	1.68	1.68×10^3
Dabcyl-GPAKLAIG-Edans[c]			
trypsin	0.040	8.1	2.0×10^5
guanyl-trypsin	0.056	20	3.56×10^5
succinyl-trypsin	0.052	4.9	9.4×10^4
Dabcyl-GPARLAIG-Edans[c]			
trypsin	0.023	25	1.1×10^6
guanyl-trypsin	0.021	70	3.31×10^5
succinyl-trypsin	0.037	70	1.88×10^5

[a] Conditions: pH 8.0 (50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$); $\vartheta = 25$°C.
[b] $\lambda = 405$ nm.
[c] $\lambda_{ex} = 336$ nm, $\lambda_{em} = 490$ nm.

3.2. Deacylation kinetics

The S′ specificity of serine and cysteine proteases can be monitored by acyl transfer reactions with added nucleophiles. This reaction is the reverse of peptide hydrolysis and, therefore, provides analogous specificity data. As indicated in Fig. 2, the acyl group of a substrate R-COX, which is usually an ester, can be transferred to both water and R′-NH2, two products are formed: R-COOH and R-CO-NHR′, respectively. The ratio between hydrolysis and aminolysis is mainly determined by the S′ specificity of the protease. The partition value p describes the acyl transfer efficiency and, therefore, it correlates with the S′ specificity [17]. p is defined according to Fig. 2:

$$p = [R' - NH_2]\, v_H / v_A = k_3 K_N / k_4, \qquad (2)$$

v_H and v_A represent the velocities of aminolysis and hydrolysis. The partition value can be determined from the product ratios when R′-NH2 is in excess:

$$p = [R' - NH_2][R - CO_2H] \,/\, [R - CONH - R'], \qquad (3)$$

where [R′-NH_2] is the initial nucleophile concentration, [R-CO_2H] and [R-CONH-R′] represent the product concentrations. The concentration of the components of the reaction mixture can be determined by several methods [18–20]. We used the UV-detection and quantification of the concentrations after HPLC.

3.3. Acyl transfer studies

To determine the influence of succinylation on the specificity of trypsin the acyl transfer of

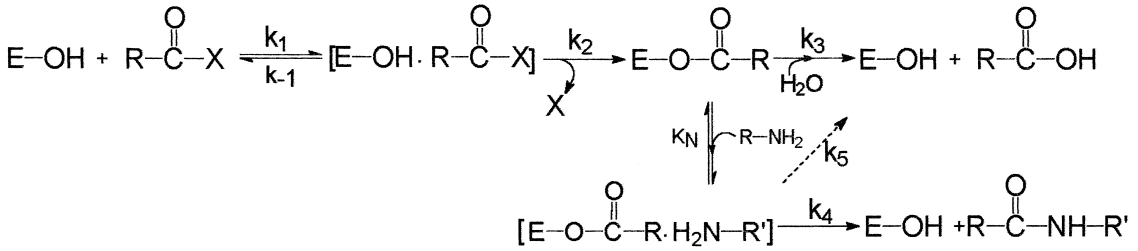

Fig. 2. Mechanism for serine protease catalyzed acyl transfer reactions. E-OH, free enzyme; R-COX, acyl donor; X, leaving group; R′-NH_2, nucleophile.

Bz-Arg-OEt on a series of amino acid amides and dipeptides was elucidated and compared with guanylated trypsin and native trypsin. As shown in Fig. 3, there are significant differences for the deacylation of Bz-Arg-(succinyl-trypsin), -(guanyl-trypsin) and -trypsin by various amino acid amides. Succinyl-trypsin displays a favourable acceptance of amino acid amides with basic residues and prefers P1′ Arg about 8-fold and P1′ Lys more than 10-fold better than native trypsin and guanyl-trypsin. This is a noteworthy result, because the substrate discrimination in the S′ binding sites is in general with up to two orders of magnitude clearly lower compared to the S1 binding pocket. The acceptance of P1′ Glu in succinyl-trypsin is decreased compared with guanyl- and native-trypsin. The uncharged nucleophiles like P1′(Ala, Gly, Leu, Met, Ser) were almost unaffected. These results are due to the change of charge of succinyl-Lys[60] which interacts with the P′1 amino acid side chain in the substrate. Succinyl-trypsin accepts basic P1′ residues (Arg, Lys) significantly better than hydrophobic aliphatic residues (Met, Leu). This is in contrast to trypsin, which prefers substrates containing aliphatic residues at P1′(Met, Leu), but also accepts aromatic (Phe) and to a lesser extend, positively charged P1′ residues (Arg, Lys) [21]. Thus, the specificity profile in succinyl-trypsin was significantly changed by the introduction of a negatively charged residue in position 60. The guanylation does not have an impressive influence on the S1′ specificity of trypsin. As expected, nucleophiles with basic residues are slightly less efficient. In general, the acceptance of P1′ residues in guanyl-trypsin is similar to native trypsin and, therefore, steric factors for the changed specificity in the modified trypsins can be excluded. The obtained results correspond to the engineering of the S1′ subsite of trypsin by site directed mutagenesis and underline the role of Lys[60] as a key determinant of the S1′ specificity in trypsin.

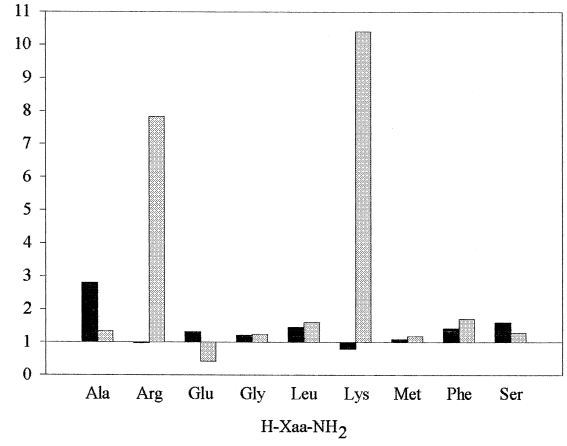

Fig. 3. Influence of guanylation and succinylation on the S1′ specificity of bovine trypsin for a series of amino acid amides: comparison of p values between trypsin and modified trypsins. Black bars: $p_{trypsin} / p_{guanyl\text{-}trypsin}$, grey bars: $p_{trypsin} / p_{succinyl\text{-}trypsin}$.

The results of the acyl transfer of Bz-Arg-OEt to different dipeptides of the general structure H-Ala-Xaa-OH are shown in Fig. 4. In all cases, the acceptance of dipeptides by guanyl-trypsin is about 3-fold better than by the native trypsin. This is probably due to the increased nucleophilicity of Har[60] and, therefore, the higher specificity for the free carboxyl group of dipep-

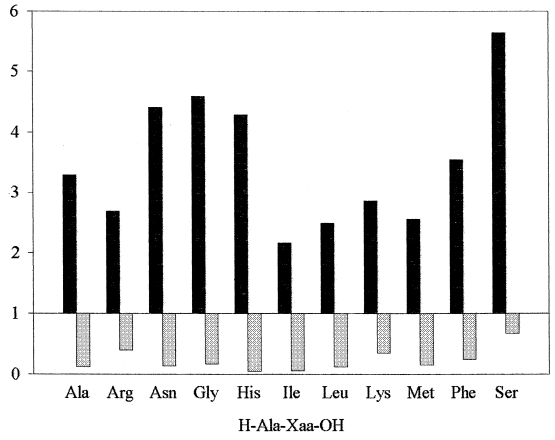

Fig. 4. Influence of guanylation and succinylation on the S2′ specificity of bovine trypsin for a series of dipeptides: comparison of p values between trypsin and modified trypsins. Black bars: $p_{\text{trypsin}} / p_{\text{guanyl-trypsin}}$, grey bars: $p_{\text{trypsin}} / p_{\text{succinyl-trypsin}}$.

tides in S2′. Accordingly, the acyl transfer reaction with succinyl-trypsin proceeds comparably worse. Generally, the profile of the S2′ specificity of amino acid side chains was not affected. Thus, the chemical modification exhibits no influence on the S2′ specificity but shows remarkable effects on the general enzyme–substrate interactions in this position.

3.4. Autolysis experiments

As shown above, lysine residues play an important role for the S′ specificity of trypsin. Moreover, they are incorporated in the regulation of enzyme activity by proteolytic cleavage. Therefore, chemical modification of lysine residues is a promising strategy to enhance the resistance against proteolytic degradation processes. With respect to the trypsinogen activation by limited proteolysis and trypsin autolysis, it is interesting to prevent these reactions. Due to autodigestion of rat trypsin, the activity of trypsin decreases by cleaving of sensitive bonds between Arg^{117}–Val^{118}, Lys^{60}–Ser^{61} and Lys^{145}–Ser^{146}. The mutations in rat trypsin K60N and, especially, R117N confer resistance against autolysis. The process of autolysis is dependent on Ca^{2+} concentration and the structure of the Ca^{2+} binding site has been identified [22]. The Ca^{2+} binding site extends from Glu^{70}

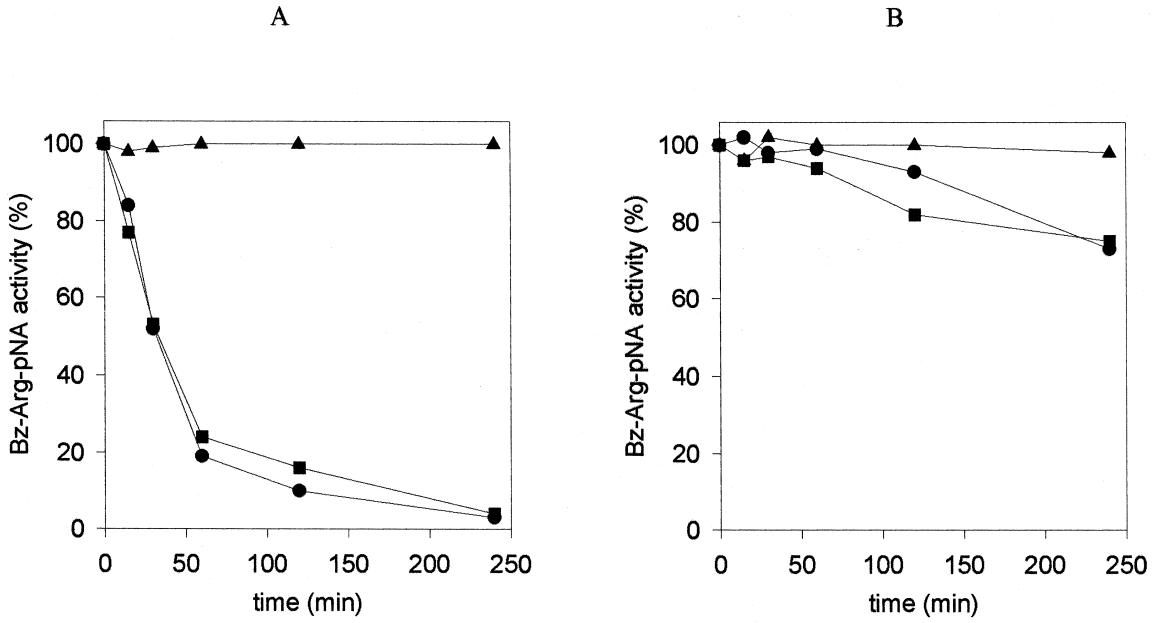

Fig. 5. Time-course of activity of bovine trypsin, guanyl-trypsin and succinyl-trypsin in absence (A) and presence of Ca^{2+} (B). (●) trypsin; (■) guanyl-trypsin; (▲) succinyl-trypsin.

to Glu^{80} in rat trypsin. It is supposed that binding of Ca^{2+} to this region can stabilize an autolysis resistant conformation between Lys^{60} and Arg^{117}. This two amino acid residues are placed in two regions, which are in neighbourhood to the Ca^{2+} binding site.

The influence of succinylation and guanylation on the autolysis of trypsin in presence and absence of Ca^{2+} is presented in Fig. 5. Generally, succinylation and guanylation leads to a selective transformation of lysine residues. Arginine residues, including Arg^{117}, should not be affected. Succinyl-trypsin displays a high stability against autodigestion. This indicates, that modification of lysine residues may increase the stability against autolytic processes. In contrast to this observation and to results known from literature [23], we have not found an increased stability of guanyl-trypsin in absence of Ca^{2+}. Therefore, we suppose that the high stability of succinyl-trypsin is probably the consequence of the negatively charged protein surface. Thus, the obtained result is in correspondence with the observation that the bond between Arg^{117}–Val^{118} is the major cleavage site during the process of autolysis.

4. Conclusions

Chemical modification of lysine residues by guanylation and succinylation leads to a significant change of S′ specificity in trypsin, whereas the catalytic behavior to standard amide substrates was not affected. The increased specificity of succinyl-trypsin towards nucleophiles with basic residues in P1′ is the most notable result obtained. This effect is probably due to the negatively charged suc-Lys^{60} in the S1′ site of trypsin which may be incorporated in electrostatic interactions with basic side chains of the deacylating substrate in P1′. These studies are in accordance to engineering the S1′ subsite by genetical substitution of Lys^{60} against Glu and underline the key role of Lys^{60} as a determinant of the S′ specificity in trypsin. Furthermore, the described chemical modifications influence the general enzyme–substrate interactions in P2′, whereas the specificity profile is not affected. The succinylation of trypsin resulted in a dramatic change of the surface charge of the protein. In trypsin, the ratio between positively charged and negatively charged amino acid residues is 15/10, in succinyl-trypsin 2/23. Therefore, we suppose that succinyl-trypsin resists against autodigestion. Our data clearly show that chemical modification is an alternative and generally an applicable tool to manipulate the specificity and stability of proteases whereas the catalytic activity is remained. With respect to the application of proteases, these studies are advantageous to improve the efficiency of biocatalysts in enzymatic peptide synthesis.

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