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Enzymatic activity and thermal stability of PEG- α -chymotrypsin conjugates

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Abstract

α -Chymotrypsin was chemically modified with methoxypoly(ethylene glycol) (PEG) of different molecular weights (700, 2000, and 5000 Da) and the amount of polymer attached to the enzyme was varied systematically from 1 to 9 PEG molecules per enzyme molecule. Upon PEG conjugation enzyme catalytic turnover (k_{cat}) decreased as much as 50% and substrate affinity was also lowered as evidenced by an increase in the Michealis-Menten constant (K_M) from 0.05 up to 0.19. These effects were dependent on the amount of PEG bound to the enzyme, but independent of the PEG size. In contrast, stabilization toward thermal inactivation depended on the PEG molecular weight with conjugates with the larger PEGs being more stable.

Keywords

biocatalyst; bioconjugate; enzyme modification; PEGylation; protein stability

Introduction

The inherent instability of proteins, both structurally and chemically, limits their use as biocatalysts and therapeutic agents, usually due to short lifetimes when subjected to physical and/or chemical stress (Wang 2005; Polizzi et al. 2007). To improve this situation, arrays of methods aimed at producing protein formulations with superior stability have been developed. For example, addition of excipients can improve liquid- and solid-phase stability of pharmaceutical proteins, mutant proteins are created with improved thermodynamic stability or lacking chemically reactive amino acids, and proteins are modified chemically to improve both *in vitro* and *in vivo* stability (Frokjaer and Otzen 2005). Perhaps, the most common chemical modification in this context is the attachment of poly(ethylene glycol) molecules to the surface of proteins, a process commonly referred to as PEGylation (Inada et al. 1995; Veronese and Mero 2008). Initially, PEGylation was carried out in a non-specific manner with heterogeneous and impure polymers but current PEGylation reagents are amino acid specific and methods have been developed to carry out site-specific modification. PEGylation has been shown to improve the stability of pharmaceutical proteins in liquid formulations (e.g., by minimizing protein-protein interactions), *in vivo* after administration by injection by reducing proteolysis, renal filtration, and loss to the immune system ("stealth" formulations), and also upon encapsulation and release from biocompatible polymer microspheres for sustained release (Castellanos et al. 2005; Veronese and Mero 2008).

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Nevertheless, PEGylation also has its drawbacks. For example, protein PEGylation is usually accompanied by a loss in activity (Pasut et al. 2008). The magnitude of this activity loss might depend on the number, location, and size of the PEG molecules attached to the enzyme. To select the most suitable PEG-protein conjugates for a particular application, it is required to systematically study the effects of the size of the PEG and the degree of modification on enzyme activity. Our study specifically addresses the latter issue by expanding the knowledge of the effect of PEGylation parameters on the enzymatic activity and thermal inactivation of the model protease α -chymotrypsin (α -CT). Proteases are important enzymes with a wide range of applications as biocatalysts and as therapeutic agents (Bordusa 2002; Stennicke et al. 2008). It has previously been shown that PEGylation increases the thermodynamic stability of α -CT (Rodríguez-Martínez et al. 2008), but information on how the PEGylation degree and the PEG molecular weight alter its activity and thermal inactivation is missing. To this effect, a series of PEG- α -CT conjugates was synthesized in which the amount of polymer conjugated to the enzyme (degree of PEGylation) as well as the size of the polymer were systematically varied.

Materials and Methods

Chemicals

α -CT from bovine pancreas and N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-4-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA) were from Sigma-Aldrich. Methoxypoly(ethylene glycol)-succinimidyl propionate (average M_W of 5,000 Da) and methoxypoly(ethylene glycol) succinimidyl- α -methylbutanoate (average M_W of 2,000 Da) were from Nektar Technologies. Methoxypoly(ethylene glycol)-N-hydroxysuccinimide ester (685.71 g/mol) and 2, 4, 6-trinitrobenzene sulfonic acid (TNBSA) were from Thermo Scientific.

Synthesis of PEG- α -CT Conjugates

Chemical modification of surface exposed lysine ϵ -amino groups of α -CT with activated PEGs of different sizes was carried out as reported (Rodríguez-Martínez et al. 2008). In brief, 100 mg of α -CT were dissolved in 100 mM borate buffer at pH 9.2. To vary the degree of modification, different amounts of activated PEGs (700, 2000, and 5000 Da) were added to the enzyme solutions. The reaction solution was stirred for 3 h at 4 °C. The reaction was stopped by lowering the pH to 5 with 1 M HCl. Unreacted PEG and buffer salts were removed by dialyzing against deionized water. The PEG- α -CT conjugates were subsequently freeze-dried and stored at -20 °C until use. The degree of protein modification was determined by colorimetric titration of unreacted amino groups with TNBSA (Habeeb 1966).

Enzymatic Activity

The enzymatic activity of α -CT and PEG- α -CT conjugates was determined by measuring the rate of hydrolysis of the substrate Suc-Ala-Ala-Pro-Phe-pNA (Solá and Griebenow 2006a). Product formation (p-nitroaniline) was followed by measuring the absorbance at 410 nm ($\epsilon_{410} = 8.8 \text{ mM}^{-1} \text{ cm}^{-1}$). All reactions were carried out in 10 mM potassium phosphate buffer (pH 7.1, 25 °C). In all reactions the enzyme concentration was 0.8 μM . To determine steady state kinetic parameters, the substrate concentration was varied from 0 to 0.5 mM. Michaelis-Menten parameters (k_{cat} and K_M) were determined by non-linear regression analysis of “initial velocity vs. [substrate]” plots.

Thermal Inactivation

α -CT and PEG- α -CT conjugates (8 μM) were incubated at 45 °C. At different times aliquots were removed, diluted by a factor of ten, and their activity determined as described previously.

The residual activity (A/A_0) was calculated as the ratio of the activity at a given time (A) over the initial activity of the enzyme (A_0).

Results and Discussion

PEGylation of α -CT

PEG- α -CT conjugates with PEG of different molecular weights (700, 2000, and 5000 Da) and varying PEGylation degree were synthesized by covalently linking activated PEG to surface accessible lysine residues of which α -CT has 14 (Tsukada and Blow 1985). Success of the procedure was verified by measuring the unreacted amino groups using the TNBSA assay. It was found that by varying the PEG-to- α -CT molar ratio in the reaction it was possible to control the degree of PEGylation of α -CT and bind from 1 up to 9 PEG molecules per α -CT molecule (Tab. 1). This corresponds to a maximum of 64% modification of the lysine residues under the conditions employed.

Enzymatic Activity of α -CT and PEG- α -CT Conjugates

All PEG- α -CT conjugates were analyzed by measuring enzyme kinetics for the hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA and the Michaelis-Menten parameters were derived (Tab. 1, Fig. 1). It was found that both the catalytic turnover rate (k_{cat}) and substrate affinity (K_M) were significantly altered by PEGylation.

The value of k_{cat} decreased at increasing amount of PEG molecules bound to the enzyme (Fig. 1A). This effect leveled off after 6 PEG molecules were bound and for the most modified α -CT the activity decreased to ~60–50% compared to the non-modified enzyme. The most likely reason for this dependency consists in increased rigidity of the enzyme upon PEGylation. Interestingly, this effect was independent of the molecular weight of PEG. Rodríguez-Martínez et al. (2008) report an increase in the T_m -value for thermal unfolding upon PEGylation and slowing in H/D exchange kinetics of the enzyme which also leveled off at around 4 to 6 PEG molecules bound. These effects were also found to be independent of the PEG M_W . Nevertheless, it has to be pointed out that we found no more than a qualitative trend when investigating the relationship between k_{cat} and protein dynamics, presumably due to complications arising from changes in the K_M -values upon PEGylation (see below).

This finding might be important to formulation scientists because it implies that it might not be possible to obtain higher bioactivities when formulating PEG-enzyme conjugates by switching from a bulky large PEG moiety to a smaller one. It is probably important to verify whether this finding is specific to α -CT or can also be found with enzymes of medical importance (e.g., PEG-asparaginase used to treat acute lymphoblastic leukemia).

Our results on the effect of the PEGylation degree on the turnover rate of α -CT agree with limited data of a previous study employing PEG5000 (Castellanos et al. 2005). A similar effect was also observed for the modification of α -CT with other of polymers. The reduction of α -CT activity upon chemical glycosylation using lactose and dextran with a M_W of 10000 Da was shown to be due to a reduction in protein structural dynamics (Solá and Griebenow 2006 a,b). The decrease in activity upon modification with poly(N-isopropylacrylamide-co-acrylamido-D-glucose) was also attributed to restricted dynamics of the enzyme after modification (Kim and Park 1999).

The dependence of the K_M -value on the amount of PEG bound to α -CT showed a dissimilar relationship (Fig. 1B). A doubling of the K_M -value was observed after the first PEG-molecules were bound and this value remained constant up to ca. 6 PEG molecules. Binding of additional PEG-molecules caused the K_M to increase further (Table 1). Similar to the effect observed for

the catalytic activity, the effect of the PEGylation degree on substrate affinity was independent of the PEG M_w .

PEG-modification of enzymes is usually accompanied by a reduction in the substrate affinity, which is in agreement with our results (Kotzia et al. 2007). The lower substrate binding affinity is due to steric crowding by the presence of PEG molecules near the substrate binding site. Since the lysine residues on the protein surface are not chemically equivalent (Solá and Griebenow 2006b), complex dependencies of the K_M on the degree of PEGylation are expected. Computational and structural studies predict that PEG molecules tend to fold and occupy a large surface area of the protein, possibly interfering with substrate binding (Manjula et al. 2003; Svergun et al. 2008).

Thermal Inactivation

A major benefit of protein PEGylation is the increased stability of the resulting PEG-protein conjugates. To maximize the benefits derived from PEGylation it is important to understand how the PEGylation parameters (i.e., PEG-size and PEGylation degree) influence protein stability. α CT and PEG- α -CT conjugates were incubated at 45 °C and the residual activity was measured at different time points. All PEG- α -CT conjugates exhibited increased stability when compared to the unmodified enzyme (Fig. 2), probably due to inhibition of autolysis due to the presence of the PEG-moiety on the protein surface (Treetharnmathurot et al. 2008). For the three PEG-sizes tested in this study a higher degree of modification resulted in more stable conjugates. α -CT lost nearly 100% of its initial activity within the first 30 minutes of incubation, whereas (PEG700) $_8$ - α -CT retained 30%, (PEG2000) $_8$ - α -CT retained 40%, and (PEG5000) $_6$ - α -CT retained 60% of their initial activity after 2.5 hours of incubation. In contrast to enzymatic activity, the PEG M_w strongly influenced α -CT thermostability. The more stable conjugates were those with the PEG of 2000 and 5000 Da. This finding might have importance to the formulation scientist – since there is no price to pay in form of decreased activity in this case when using large PEG-molecules, for α -CT PEG5000 would probably be the best choice.

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Fig 1. Effect of PEG-size and PEGylation degree on α -CT kinetic parameters, k_{cat} (A) and K_M (B). α -CT (solid circle), PEG700- α -CT (open circles), PEG2000- α -CT (solid squares), and PEG5000- α -CT (open squares).

Fig 2.

Thermal inactivation of α -CT and PEG- α -CT conjugates for PEG of 700 (A), 2000 (B), and 5000 Da (C). Figure 2A: α -CT (solid circle), (PEG700) $_4$ - α -CT (open circle), and (PEG700) $_8$ - α -CT (solid square). Figure 2B: α -CT (solid circle), (PEG2000) $_1$ - α -CT (open circle), (PEG2000) $_4$ - α -CT (solid square), (PEG2000) $_8$ - α -CT (open square). Figure 2C: α -CT (solid circle), (PEG5000) $_1$ - α -CT (open circle), (PEG5000) $_3$ - α -CT (solid square), (PEG5000) $_4$ - α -CT (open square), (PEG5000) $_6$ - α -CT (solid triangle).

Table 1PEGylation degree and Michaelis-Menten kinetic parameters for PEG- α -CT conjugates.^a

Sample	Degree of PEGylation ^b	k_{cat} (s ⁻¹) ^c	K_M (mM) ^c
α -CT (unmodified)	none	12.1 $\pm$ 0.9	0.05 $\pm$ 0.01
PEG700- α -CT			
	3.8 $\pm$ 0.2	11.6 $\pm$ 0.4	0.07 $\pm$ 0.01
	7.9 $\pm$ 0.4	6.2 $\pm$ 0.6	0.15 $\pm$ 0.01
PEG2000- α -CT			
	1.3 $\pm$ 0.6	13.5 $\pm$ 0.4	0.08 $\pm$ 0.04
	2.2 $\pm$ 0.2	10.7 $\pm$ 0.4	0.07 $\pm$ 0.01
	4.2 $\pm$ 0.3	7.6 $\pm$ 0.5	0.09 $\pm$ 0.01
	8.4 $\pm$ 0.4	9.4 $\pm$ 0.5	0.15 $\pm$ 0.01
	9.1 $\pm$ 0.3	7.9 $\pm$ 0.2	0.19 $\pm$ 0.02
PEG5000- α -CT			
	1.3 $\pm$ 0.3	13.1 $\pm$ 0.5	0.10 $\pm$ 0.01
	2.9 $\pm$ 0.3	10.2 $\pm$ 0.5	0.08 $\pm$ 0.01
	4.2 $\pm$ 0.4	8.7 $\pm$ 0.5	0.08 $\pm$ 0.01
	6.0 $\pm$ 0.6	8.3 $\pm$ 0.4	0.08 $\pm$ 0.02
	7.6 $\pm$ 0.7	8.6 $\pm$ 0.5	0.11 $\pm$ 0.01

^a Values are the average of three measurements $\pm$ SD.^b Average mol of PEG polymer per mol of α -CT.^c Determined by non-linear regression of "initial velocity vs. [substrate]" plots.