Amino Group PEGylation of Bovine Lactoferrin by Linear Polyethylene Glycol-\(p\)-nitrophenyl Active Esters

Kanako Kato, Naomi Tamaki, Yoshiki Saito, Tomohito Fujimoto, and Atsushi Sato*

School of Bioscience and Biotechnology, Tokyo University of Technology; 1404–1 Katakura, Hachioji, Tokyo 192–0982, Japan. Received November 23, 2009; accepted April 25, 2010; published online April 28, 2010

PEGylation, the covalent attachment of polyethylene glycol (PEG) to pharmaceutical proteins, is regarded as an extremely useful procedure to generate protein drugs with intensified therapeutic properties. We examined the amino group modification of bovine lactoferrin (bLF) with linear PEG-\(p\)-nitrophenyl active esters. At pH 5.0, we specifically observed the formation of mono-PEGylated bLF in high yields. PEG-conjugation reactions advanced slowly and reached a steady state by 48 h in a buffer at pH 5.0. The hydrolysis half-lives of 5-kDa and 30-kDa PEG-\(p\)-nitrophenyl active esters at pH 5.0 were estimated to be approximately 117 and 136 h, respectively. The slow reaction and hydrolysis rates of PEG-\(p\)-nitrophenyl active esters may contribute to the formation of mono-PEGylated bLF that could not be obtained by PEGylation with linear \(N\)-hydroxysuccinimide (NHS) activated PEG.

Key words: \(p\)-nitrophenyl active ester; polyethylene glycol; pH; kinetic; lactoferrin; hydrolysis

Lactoferrin (LF), an 80-kDa member of the transferrin family of iron-binding glycoproteins, is found in most mammalian body fluids and exocrine secretions such as milk, tears, saliva, and intestinal secretions.\(^{11}\) It can be regarded as an important immunomodulatory protein that links the innate and adaptive immune functions by regulating target cell responses.\(^{21}\) Talactoferrin\(^{5}\), a recombinant human LF, was reported to be effective in the treatment of patients with diabetic neuropathic foot ulcers\(^{3}\) and in non-small-cell lung cancer (NSCLC) therapy in combination with cisplatin treatment in phase I/II clinical trials.\(^{4}\) Talactoferrin\(^{5}\) has also been used in a recent clinical trial for renal cell carcinoma.\(^{5}\) Thus, LF is a potential biopharmaceutical agent. Recently, we developed two conjugates by PEGylation of bovine LF (bLF) with 20-kDa and 40-kDa 2-branched poly(ethylene glycol) (PEG).\(^{6,7}\) PEGylated bLF possessed high levels of biological activity and exhibited enhanced pharmacokinetic properties. Compared to the intraperitoneal administration of native bLF, the intraperitoneal administration of PEGylated bLF in rats enhanced the hepatoprotective effects on acute liver injury induced by carbon tetrachloride and by \(\beta\)-galactosamine and lipopolysaccharide.\(^{8,9}\) PEGylation of proteins is an established method for improving the pharmacokinetic properties (e.g., reduction of renal ultrafiltration by increasing the molecular size of proteins and improvement of their stability against proteolytic cleavage by creating a molecular shield of PEG around them) and pharmacodynamic effects of therapeutic proteins.\(^{10}\) The amino groups of proteins are often used as substrates for PEGylation because they are the most represented groups in proteins and are highly reactive.\(^{11}\) \(N\)-Hydroxysuccinimide (NHS) active esters of PEG (PEG-NHS) are used most frequently for amino group modification of target proteins. NHS-active esters produce stable amide linkages between PEG and primary amines such as amine residues. In order to synthesize linear PEG-conjugated bLF, we first chose linear PEG-NHS as active PEG derivatives. However, despite the extensive analysis of PEG reactions by varying pH, protein concentrations, and PEG-to-bLF ratios, linear PEG-NHS with average molecular weights of 5 kDa and 30 kDa could not be used for the production of mono-PEGylated bLF as judged from smear patterns on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. This phenomenon may have occurred due to the existence of 54 internal lysine residues that act as potential conjugation sites in bLF and also due to the high reaction rates of PEG-NHS. We next attempted to use PEG-\(p\)-nitrophenyl active esters which are known as slowly reactive PEG derivatives.\(^{11}\) This led to the successful production of mono-PEGylated bLF. In this study, we investigated the kinetics of the conjugation reaction between PEG-\(p\)-nitrophenyl active esters and bLF and the hydrolysis rates of PEG-\(p\)-nitrophenyl active esters. Comparison of the kinetics and hydrolysis rates of PEG-\(p\)-nitrophenyl active esters with those of PEG-NHS is discussed. The present investigation suggests that the slow reaction and hydrolysis rates of PEG-\(p\)-nitrophenyl active esters may contribute to the formation of mono-PEGylated bLF.

MATERIALS AND METHODS

Optimal pH Conditions for PEGylation of Bovine Lactoferrin (bLF) Two kinds of linear PEG-\(p\)-nitrophenyl active esters with average molecular weights of 5 kDa (SUNBRIGHT MENP-50H, NOF Corp., Tokyo, Japan) and 30 kDa (SUNBRIGHT MENP-30T, NOF Corp., Tokyo, Japan) were used in this study. PEGylated reaction mixtures contained bLF (MG Nutritionals, Melbourne, Australia) and each PEG-\(p\)-nitrophenyl active ester at a 1:5 molar ratio. The reaction mixtures were dissolved in the following buffers at the indicated pH: 50 mM acetate buffer (pH 4.0, 5.0); 50 mM phosphate buffer (pH 6.0, 7.0, 8.0); or 50 mM borate buffer (pH 9.0). The final bLF concentration was 0.5 mg/ml. Reactions were carried out for 24 h at 25 °C and stopped by the addition of SDS-PAGE loading buffer. The reaction mixtures (corresponding to 2 μg bLF) were then subjected to 7.5% SDS-PAGE under nonreducing conditions and visualized by Coomassie Brilliant Blue (CBB) staining.

Conjugation Reaction Kinetics of Linear PEG-\(p\)-nitrophenyl Active Esters PEGylated reaction mixtures contained bLF and each PEG-\(p\)-nitrophenyl active ester at a 1:1
molar ratio in 50 mM acetate buffer (pH 5.0). The final bLF concentration was 15 mg/ml. Conjugation reactions were carried out for indicated times at 25 °C and stopped by the addition of the SDS-PAGE loading buffer. Reaction samples (corresponding to 2 μg bLF) taken at indicated times were subjected to 7.5% SDS-PAGE under nonreducing conditions and visualized by CBB staining. After CBB staining, the intensities of CBB-stained bands were analyzed by Image J software and represented as graphs.

Hydrolysis Half-lives of Linear PEG-p-nitrophenyl Active Esters Conjugation reactions were carried out as described above except that each PEG-p-nitrophenyl active ester was preincubated in 50 mM acetate buffer at pH 5.0 for the indicated times and then mixed with bLF to start the coupling reactions (the final bLF concentration was 15 mg/ml with a PEG-to-protein molar ratio of 1 : 1). PEGylation was carried out for 48 h in 50 mM acetate buffer at pH 5.0 and 25 °C. Reactions were stopped by the addition of the SDS-PAGE loading buffer. Reactants (corresponding to 2 μg bLF) were analyzed by 7.5% SDS-PAGE under nonreducing conditions and visualized by CBB staining. The intensities of CBB-stained bands were analyzed by Image J software and represented as graphs.

RESULTS AND DISCUSSION

pH-Dependent Conjugation of bLF with Linear PEG-p-nitrophenyl Active Esters We investigated the conjugation of two linear PEG-p-nitrophenyl active esters with average molecular masses of 5 kDa and 30 kDa to bLF at various pH values. The reaction formula for the PEGylation of bLF is illustrated in Fig. 1. Figure 2 shows the SDS-PAGE profile of PEGylation reaction mixtures in various buffers at different pH values. Regardless of the molecular mass of the PEG, very similar reaction patterns were observed. The bLF was barely PEGylated at pH 4.0 and 6.0. The mono-PEGylated bLF was formed with oligo-PEGylated bLF (as indicated by asterisks) and/or low molecular weight byproducts at pH 7.0 and above (as indicated by gray arrows). The formation of mono-PEGylated bLF in high yields was specifically observed at pH 5.0. It should be noted that mono-PEGylated bLFs migrated on SDS-PAGE with higher apparent molecular masses than the actual ones because of the contribution of the hydrated PEG moieties to their hydrodynamic radii. PEG-NHS, the most frequently used PEG derivatives, typically couples with the free amino group of the targeted protein at a physiological pH range of 7.0 to 9.0, and the PEGylated protein is barely formed under acidic conditions (pH 4—6). On the other hand, by using PEG-p-nitrophenyl active esters, we observed the formation of mono-PEGylated bLF in high conjugation yields at pH 5.0. Therefore, PEG-p-nitrophenyl active esters with an optimal pH that is more acidic than that of PEG-NHS could be used for amino conjugation of proteins under acidic conditions.

Kinetic Analysis of PEGylated bLF with Linear PEG-p-nitrophenyl Active Esters at pH 5.0 Next, we compared the kinetics of conjugation reactions carried out with linear PEG-p-nitrophenyl active esters having different molecular masses. The reaction products were analyzed by SDS-PAGE, as shown in Figs. 3A and B. The intensities of the CBB-stained bands were analyzed by Image J software (Fig. 3C). The reactions involving 5-kDa and 30-kDa linear PEG-p-nitrophenyl active esters proceeded up to 48 h, with reaction curves reaching a plateau thereafter. The reaction velocities of PEG-p-nitrophenyl active esters were much lower than those of PEG-NHS. PEGylation reactions with linear PEG-p-nitrophenyl active esters reached a steady state by 48 h at pH 5.0 and 25 °C, whereas those with 2-branched PEG-NHS active esters reached a steady state by 2 h at pH 7.4 and 25 °C and by 10 min at pH 9.0 and 25 °C.

Hydrolysis Half-lives of Linear PEG-p-nitrophenyl Active Esters at pH 5.0 The reaction rate of PEG-p-nitrophenyl active esters is the result of the balance between the gain in reaction velocity and the loss of PEG-p-nitrophenyl active esters by hydrolysis. Therefore, we determined the hydrolysis half-lives of linear 5-kDa and 30-kDa PEG-p-nitrophenyl active esters at pH 5.0 (Fig. 4). The hydrolysis half-lives of 5-kDa and 30-kDa PEG-p-nitrophenyl active esters at pH 5.0 were estimated to be approximately 117 and 136 h, respectively. The hydrolytic stabilities of PEG-p-nitrophenyl active esters were much greater than those of PEG-NHS. The hydrolysis half-lives of 5-kDa and 30-kDa PEG-p-nitrophenyl active esters were approximately 117 and 136 h, re-

![Fig. 1. Reaction Scheme of Synthesis of PEGylated Bovine Lactoferrin (bLF)](image)

A linear polyethylene glycol (PEG)-p-nitrophenyl active ester yields an amide linkage with the α- or ε-amino group of bLF.
respectively, at pH 5.0 and 25 °C, whereas those of 20-kDa and 40-kDa 2-branched PEG-NHS were estimated to be approximately 128 and 166 min, respectively, at pH 7.4 and 25 °C and approximately 9.0 and 5.0 min, respectively, at pH 9.0 and 25 °C.13)

In summary, we have successfully obtained PEGylated bLF with linear PEG-p-nitrophenyl active esters at pH 5.0. There was no significant difference in the reaction and hydrolysis rates between 5-kDa and 30-kDa PEG derivatives. Because PEG-p-nitrophenyl active esters showed much lower reaction velocities and much greater hydrolytic stabilities than PEG-NHS, these factors may contribute to the formation of mono-PEGylated bLF.

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