Coupling of Monomethoxypolyethyleneglycols to Proteins *via* Active Esters

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Two alternative methods for the attachment of monomethoxypolyethyleneglycols (PEG) to proteins are proposed; they are based upon the replacement of the hydroxy terminal function of PEG to carboxylate followed by its activation with dicyclohexylcarbodiimide and N-hydroxysuccinimide. The methods, which give more homogeneous product than that employing trichloro-s-triazine as coupling reagent, may also be used for the modification of essential –SH containing enzymes. The attachment of PEG activated *via* esters was tested with several model proteins and the influence of the extent of modification i. on the biological activity of various enzymes, ii. on the binding capacity for albumin and iii. on the clearance time in rats using superoxide dismutase as model tracer was evaluated. It was also demonstrated that the extent of PEG attachment varies greatly according to the different proteins used.

Introduction

Coupling of soluble polymers to the protein surface appeares a promising method in obtaining more situable derivatives for medical application. Poly-(N-vinylpyrrolidone) [1, 2], monomethoxypolyethyleneglycol (PEG) [3–8], homologous albumin [9], dextranes [10–12], have mainly been used as biocompatible polymers. The modified enzymes often have useful properties such as increased stability to proteolytic digestion, higher half life time when injected into the blood and reduced immunological response. As a contribution to these studies we recently evaluated the pharmacokinetic behaviour of superoxide dismutase when modified to different degrees with monomethoxypolyethyleneglycols of different lengths [13]. During the investigation we found certain disadvantages in coupling the polymer through trichloro-s-triazine according to the method reported in the literature (3): a. the modified enzyme presents species at higher molecular weight than expected on the base of bound PEG; this probably due to the fact that the third chloride of the reagent reacted further giving a cross-linked protein; b. the modification is unsuitable for enzymes possessing reactive –SH groups in the active site; and c. the modified enzyme presents significant absorption in the UV region due to the s-triazine ring and, therefore, spectroscopic characterizations are greatly hampered. Furthermore, the presence of chlorotriazine derivatives in compounds for clinical use is dangerous according to the regulations given by Food and Drugs Administration.

On the basis of these considerations, we studied a new method of PEG coupling which involves the conversion of the ω-carbon of the polymer to the carboxylic group followed by coupling to proteins lysine residues *via* active esters.

We here report results obtained with the preparation of activated PEG of different mol wt by two methods, their coupling to model proteins and the chemical and pharmacokinetic characterization of samples of a derivatized enyzme.

Materials and Methods

Materials. Bovine serum albumin, fraction V, erythrocyte superoxide dismutase, bovine liver catalase and trinitrobenzene sulfonic acid were supplied by Sigma, crystallin mercuripapain and trypsin by Worthington, and yeast 6-phosphogluconate dehydrogenase by Boehringer. Monomethoxypolyethyleneglycols of 1900 and 5000 daltons (PEG 1900 and 5000) were supplied by Union Carbide; Bio-Gel A-0.5 m was obtained from Bio-Rad, 2,4,6-trichloro-s-triazine from Aldrich. Tritium labelled psoralen, specific activity 8.48×10^3 dpm/µg was prepared as described elsewhere [14]. Manganese dioxide and other reagents and salts were supplied by Fluka.

Methods. Enzyme assays of superoxide dismutase were carried out according to McCord and Frido-

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vich's method [15]. Trypsin and papain activities were evaluated with a pH-stat using as substrates *p*-toluenesulfonyl-L-arginine methyl ester, benzoyl-L-arginine ethyl ester or heat denatured albumin. Catalase activity was evaluated according to the method of Beers and Sizer [16] and 6-phosphogluconate dehydrogenase as reported elsewhere [17].

Protein concentrations were estimated from the absorption at 280 nm and, in extensively modified samples, by amino acid analysis. The extent of the amino groups modification was evaluated by the trinitrobenzenesulfonic acid method [18]. Equilibrium dialysis experiments were performed as reported elsewhere [14]. Plasma clearance evaluation of native and modified SOD was performed in Levis male rats (200–250 g) injected with 8 mg/kg of native enzyme or equimolecular amounts of modified samples in 0.2 ml of salt solution into the saphena vein. Blood was collected from the tail vein on a schedule time which varied with the SOD species centrifuged and assayed for enzymatic activity.

Direct oxidation of the primary alcoolic function of PEG to the corresponding carboxylic acid (PEG-COOH)

Method A. To activated manganese dioxide [19, 20], 10 g, PEG-5000, 10 g, in 100 ml of dry CH_2Cl_2 were added and stirred overnight at room temperature. The suspension was filtered and the solvent removed by evaporation. The dry polymer was dissolved in 200 ml of 3% hydrogen peroxide and left standing overnight at room temperature. The solution was directly applied to an AG 1 × 1 column (2.5 × 20 cm) which was rinsed throughly with water to remove the polymer still carring the unoxidized carbinol group as well as the intermediate aldehyde. The PEG-COOH was finally eluted with HCl N/50 and liophylized, the yield was 6.5 g. The complete convertion to carboxylate was further verified by micropotentiometric titration of the polymer with NaOH. The same procedure was used for PEG 1900 and gave similar yields.

Conversion of the terminal hydroxy function of PEG to amino group (PEG–NH_2) and its reaction with succinic anhydride (PEG–C_2–COOH)

Method B. A procedure similar to that described by Pillai *et al.* [21] for the conversion of the two terminal hydroxyl groups of polyethyleneglycols was used for the replacement of the single terminal hydroxy group of monomethoxypolyethyleneglycol, PEG 5000. The product (PEG–NH_2) purified by crystallization from ethanol was further purified by adsorption to an AG 50WXI column eluted with ammonium hydroxide solution N/50. The overall yield was 60%. The dry PEG-NH_2, 10 g, was dissolved in 100 ml of anhydrous dioxane, added of 5 g of succinic anhydride and, after standing overnight at room temperature precipitated with dry ether. The polymer, PEG–NH–CO–CH_2–CH_2–COOH, (PEG–C_2–COOH), was purified with ion exchange resin as reported above. A micropotentiometer titration confirmed the identity of the product. With the same procedure was prepared PEG–C_2–COOH 1900.

Activation of PEG–COOH or PEG–C_2–COOH via active ester. The dry polymer, 2 mmol, prepared according to method A or method B, were dissolved in 100 ml of dry dimethylformamide (DMF) cooled to 4 °C, 50 mmol of N-hydroxysuccinimide were added, and finally 50 mmol, of dicyclohexylcarbodiimide in DMF were added dropwise. The solution was left overnight under constant stirring to react. Urea was removed by filtration and the polymer precipitated with dry ether, was taken up which CH_2Cl_2 and recrystallized from this solution by dropwise addition of ether with rapid stirring, the mixture being kept in an ice bath. The white flaky product (overall yield of 80%) was filtered, washed and dried under vacuum.

Covalent binding of activated PEG to proteins. The protein solution (usually 20 mg in 10 ml of 0.1 M borate buffer pH 8.2) was added of activated PEG–COOH or PEG–C_2–COOH, in molar excess over the available amino groups (Tables I and II) while the pH was maintained with a pH state. After standing for 6 h at 4 °C the solution was divided into three portions and passed through a Bio-gel A-0.5 m (3 × 140 cm) equilibrated with 0.1 M phosphate buffer pH 7.2. Protein containing fractions (OD 280 nm) were pooled and the solution directly used for the characterization and evaluation of biological activity. Lyophylization was usually found to be a suitable method for long storage of the modified proteins.

Results

Derivatization of proteins with activated PEG. Table I and II report the results obtained with five

Table I. Conditions for the preparation and properties of PEG-protein derivatives prepared according to method A, (activated PEG–COOH), at various extent of modification.

Protein	Activated PEG over protein in the reaction mixture (mol/mol)	Per cent of lysine residues modified in the protein molecule	PEG linked to protein (mol/mol)	Approximate mol wt of the PEG protein[a]	% Remaining enzymatic activity or binding properties[c]
Bovine serum albumin (mol wt 66 000)	180	63	38	256 000	19 ± 3[c]
Bovine serum albumin	600	85	51	380 000	21 ± 3[c]
Catalase (mol wt 252 000)	325	56	60	540 000	95 ± 5
Trypsin (mol wt 23 800)	54	83	15	99 000[b]	110 ± 5
Papain (mol wt 23 000)	27	33	3	38 000	80 ± 5
Superoxide dismutase (mol wt 31 200)	10	15	3	46 000[b]	90 ± 5
	60	35	7	66 000[b]	80 ± 5
	200	90	18	121 000[b]	72 ± 5

[a] The mol wt was calculated from the number of modified lysine residues and, in same samples [b] also by gel filtration.
[c] The binding property, verified by equilibrium dialysis with tritiated psoralen as ligand, is referred to the binding of native albumin. The dialysis was performed at 5 mg/ml of albumin or equimolar amount of derivatized albumin and psoralen 1 μg/ml in 0.1 M phosphate buffer pH 7.4.

Table II. Conditions for the preparation, and properties of PEG-protein derivatives prepared according to method B, (activated PEG–C_2–COOH), at various degrees of modification.

Protein	Activated PEG over protein in the reaction mixture (mol/mol)	PEG linked to protein (mol/mol)	Per cent of lysine residues modified in the protein molecule	Approximate mol wt of the PEG protein[a]	Remaining enzymatic activity or binding properties[c]
Bovine serum albumin	180	36	60	246 000[b]	20 ± 3[c]
	600	53	88	330 000	–
Superoxide dismutase	10	3	13	46 000	90 ± 5
	60	6	30	61 000	80 ± 5
	800	18	90	121 000	70 ± 5

[a] The mol wt was calculated from the number of modified lysine residues and also [b] by gel filtration.
[c] The binding was evaluated as reported in Table I.

proteins with different molecular size and amino acid composition, used as models in the coupling of PEG to proteins *via* active esters. The coupling was verified more extensively with PEG–COOH prepared by oxydation (method A) because of its convenient synthesis.

The Tables show: i) the extent of amino groups modifications at different reagent to proteine lysine residues molar ratios, ii) the molecular weight of the derivatives calculated on the basis of the extent of amino groups modified, iii) the enzymatic activity of the modified enzymes, iv) the variation of binding capacity evaluated by equilibrium dialysis, using titriated psoralen as the ligand in the case of albumin.

The tables show that with the same reagent to lysine residues molar ratio the same degree of modification is observed with the polymers activated according to method A or B. They also demonstrate that, as verified with superoxide dismutase, samples modified to the same degree by the two reagents present the same loss of enzymatic activity.

With most of the samples the homogeneity of the products was assessed by gel filtration: in each case, symmetrical peaks of elution were obtained and

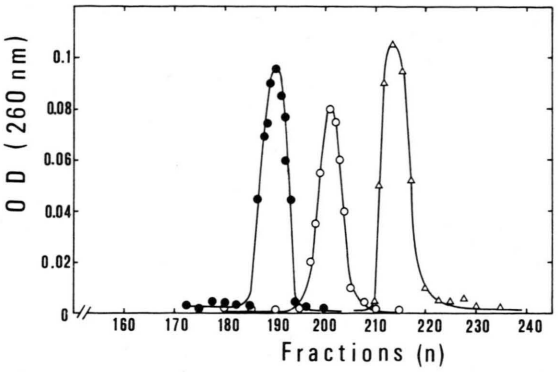

Fig. 1. Elutions of superoxide dismutase (△—△) and of the derivatives containing 7 (○—○) and 18 (●—●) molecules of bound PEG-5000 per enzyme molecule respectively. A column of Bio Gel A 0.5 m (140 × 3 cm) was used with potassium phosphate buffer 0.1 M pH 7.2: 10 mg of native enzyme or equimolecular amounts of derivatized samples were separately chromatographed and fractions of 3.4 ml were collected.

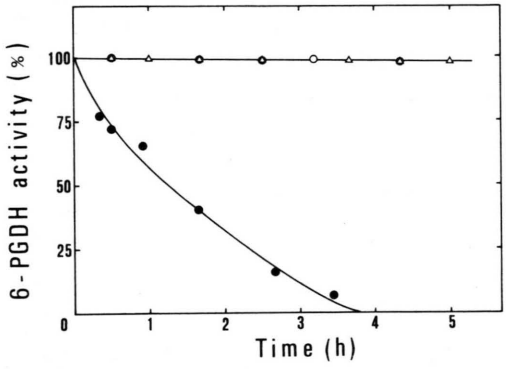

Fig. 2. Time course of inactivation of yeast 6-phosphogluconate dehydrogenase during the coupling with PEG-5000 activated *via* trichloro-s-triazine (●—●) and *via* active ester according to method A (△—△) and B (○—○) The experiments of derivatization were performed under nitrogen flushing in 0.1 M borate buffer pH 8.2; a molar ratio of activated PEG over protein of 3:1 was used in all cases.

absence of polymerized material as can be seen in Fig. 1 which gives the elution profiles of native superoxide dismutase and of samples modified at seven and eighteen lysine residues.

Derivatization of enzymes with essential SH groups. Fig. 2 reports the time course of inactivation of yeast 6-phosphogluconate dehydrogenase, an enzyme possessing an essential −SH group at the active site [22], in the presence of PEG activated *via* trichloro-s-triazine. The inactivation can be explained on the basis of its reactivity towards −SH groups as already verified by Wieder *et al.* with phenylalanine ammonia-liase [6]. On the other hand the enzyme is stable in the presence of PEG activated *via* esters (see figure) and a derivative containing approximately 15 mol of PEG per enzyme molecule still possessing 85% of its activity was obtained when a molar ratio of reagent over lysine of 3 to 1 was used in the modification.

These results are also compatible with the limited loss of activity of the modified papain (see Table I) which also possesses an essential −SH group in the active site.

We also found that papain could be modified with significant retention of the activity, using PEG activated *via* trichloro-s-triazine provided the mercury derivative of the enzyme was used. In this case, the mercury papain (28 mg suspended in 5 ml of borate buffer 0.1 M pH 8.2) was added of activated PEG (167 mg, a ratio of 3 mol of PEG per lysine residue), and left for 3 h at room temperature to react. The modification was accompanied by solubilization of the protein. The modified protein was purified on a Bio-Gel A 0.5 column. The product, which presented six molecules of PEG linked per protein molecule, recovered about 45% of the native papain activity upon removal of the mercury with excess cysteine.

Pharmacokinetic behaviour of PEG derivatized superoxide dismutase. The influence of the modification on the clearance time was studied in animals using

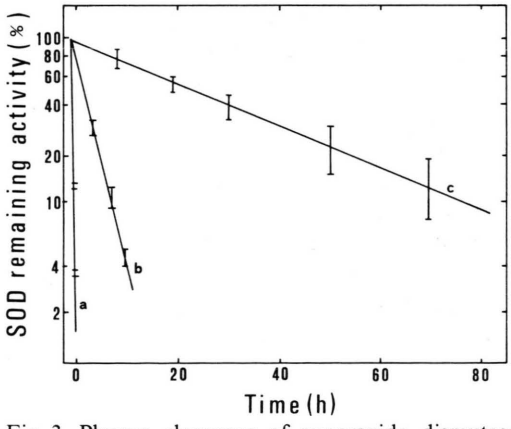

Fig. 3. Plasma clearance of superoxide dismutase enzymatic activity following intravenous injection of native and PEG-modified species into rats ($n = 3$). 100% indicates SOD activity at the end of the injection. (a) native enzyme and SOD-PEG derivatives with 3 (b) and 18 (c) molecules of PEG linked per enzyme molecule respectively.

as the model superoxide dismutase an enzyme which is very rapidly cleared in rats at the native state [23] while it is slowly eliminated when modified with PEG linked through the triazine ring [7, 13]. This enzyme was chosen for its interest as a new antiinflammatory agent [23].

Fig. 3 reports the clearance of the unmodified enzyme and of samples modified to different extents by the method A proposed here.

Half lives of 4 and 20 h were obtained for the SOD-PEG derivatives with 3 and 18 mol of PEG linked per enzyme molecule which correspond to mol wt of 46000 and 121000 Daltons respectively.

Discussion

The advantages offered by PEG in the modification of enzymes for therapeutic use, such the decrease of clearance time of the derivatized enzyme as well as the decrease in immunological response, prompted us to improve the method of polymer coupling since those so far proposed, which employ trichloro-s-triazine as the reagent have disadvantages.

The monomethoxypolyethyleneglycols still appeared to be the most suitable starting polymers, since they are commercially available in pure form and at various sizes.

The coupling of the polymer *via* active esters, once a carboxylate function could be introduced, appeared the most convenient method for its specificity because only the nucleophile-NH_2 in a protein at pH 8.2 gives rise to a stable covalent bond (amide bond). Furthermore, unlike with the trichloro-s-triazine, the reaction can be better carried out at neutral pH values.

For the convertion of the hydroxyl function of PEG to carboxylate we first considered the simple reaction with succinic anhydride, but this procedure was discarded since an ester bond would be introduced: this is known to be very easily hydrolyzed both spontaneously under physiological pH or enzymatically by unspecific hydrolases present in the blood [24].

We used therefore two alternative methods one, more troblesome, which implies the conversion of the terminal hydroxyl groups to amino followed by reaction with succinic anhydride to give a stable amide bond and a free carboxy groups, and a second which gives the carboxyl function by a two steps oxydation of the carbinol; in both cases soluble DCCI and N-hydroxysuccinimide were finally used for the preparation of the active ester.

The polymers, modified according to the two methods, were linked to several model proteins giving products which, differing in the chain length by about 5 Å, were indistinguishible with regard to yield and residual activity when modified to the same extent. A comparative study with various proteins showed that the extent of amino group modified depends largely upon the protein structure, and furthermore that for the same protein the extent of modification is not linear with the amount of reagent: the modification of the first lysines occurs at a much lower molar excess of reagent.

Experiments with 6-phosphogluconate dehydrogenase and papain demonstrated that also enzymes with reactive SH may be derivatized without appreciable loss of activity.

The modified proteins were found to be homogeneous and, the high mol wt species obtained (probably protein cross-linked through the two reactive chlorides of activated PEG [13]) when samples were derivatized with PEG *via* trichloro-s-triazine were not detected.

With superoxide dismutase as model we could demonstrate that also *in vivo* the PEG-protein obtained according to the method that we propose behaves as a stable derivative. The large increase in the half-life of the protein upon derivatization is the same that we previously found when PEG was bound *via* trichloro-s-triazine [13].

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