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Acylation of Amino Functions of Proteins with Monomethoxypoly (ethylene glycol)-*N*-Succinimide Carbonate

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ABSTRACT

Monomethoxypoly(ethylene glycol)-*N*-succinimide carbonate (SC-PEG) was used to prepare PEG–lysozyme, PEG–papaya proteinase III, PEG–catalase, and PEG–lactoperoxidase conjugates. SC-PEG produced extensively modified enzymes under mild conditions (pH 7.0; 25°C) within a couple of hours. PEG–enzyme conjugates showed equal or even greater specific activity provided that low-molecular-weight substrates were used to evaluate the biological activities. However, papaya proteinase III and lysozyme lost their proteolytic and bacteriolytic activities, respectively, on conjugation with PEG. This was most probably because of steric factors, since no drastic conformational changes could be detected after conjugation of these enzymes with PEG chains. Unlike these enzymes, the secondary structures of the two hemoproteins were somewhat affected by the covalent attachment of PEG chains as shown by FTIR experiments. These results confirmed the potential usefulness of SC-PEG, for which a novel route of synthesis making use of *N,N'*-disuccinimidyl carbonate was described.

Index Entries: mPEG; covalent attachment; lysozyme; catalase; lactoperoxydase; papaya proteinase.

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INTRODUCTION

PEGs constitute a family of polymers that have recently generated a renewal of interest. This is because numerous observations that show that the unique properties of PEGs could be conferred to materials to which these polymers are covalently bound. In several cases, this approach has yielded new applications for these materials in the important fields of medicine and biotechnology.

Molecules bound to PEG have altered solubility properties. Therefore, PEG attachment has been used to improve water solubility. Similarly, PEG binding can improve the solubility of proteins in organic solvents wherein some of them retained biological activity (*1–7*). On the other hand, PEG in aqueous solution acts as a highly mobile molecule with a large exclusion volume. Interesting consequences of this property are that PEG excludes other hydrophilic polymers, including proteins. Thus, the covalent attachment of PEG to surfaces greatly retards protein adsorption to these surfaces (*8*) and prevents surface-induced platelet activation (*9*). In this context, PEG has also been used to coat hydrophobic chromatographic supports to obtain ligands having a reduced hydrophobicity, which led to concept of mild hydrophobic chromatography (*10*).

Repulsion of other hydrophilic polymers, such as dextran, leads to the formation of two aqueous polymer phase systems in which biological materials can be partitioned. Because of their affinity for the upper phase of PEG–dextran two-phase systems, PEG–protein conjugates have proven to be useful as diagnostic tools and as means for separating different types of cells (*11–14*). Nevertheless, the most promising applications of PEG–polypeptide conjugates undoubtedly arise from their therapeutic use as drugs. Many problems encountered in the course of the delivery of such material may indeed be alleviated by its conjugation to PEG.

Proteins modified with PEG have altered pharmacokinetics. Their renal clearance rates are reduced, and they show a prolonged circulatory life in the blood vessels. Their antigenicity and their immunogenicity are reduced and even, in some cases, suppressed (*15,16*). Covalent modification of proteins with PEG may also induce increased uptake of the polypeptides by various types of cells (*17,18*).

Enzymes to which PEG is attached usually remain active toward small substrates, thereby demonstrating that bound PEG does not denature proteins or hinder the approach of small molecules. Bound PEG does, however, retain its ability to repel other large molecules. Consequently, there are few examples of PEG–enzymes with large substrates that remain fully active.

Several methods of PEG activation for coupling to proteins are reported in the literature. Most of these methods involve the preparation of an active PEG intermediate with a functional group possessing reactivity toward amino groups in proteins.

The cyanuric chloride approach has various disadvantages, such as the toxicity of cyanuric chloride and the absence of selectivity shown by the activated PEG toward the amino functions of the protein. The modification of proteins with cyanuric chloride-activated PEG is often accompanied by a substantial loss of biological activity (*19*). This is also the case for PEG chloroformates (*20*). At the opposite end of the scale, carbonylimidazol-1-yl-PEG has rather mild reactivity, and the proteins modified by this method usually exhibit good preservation of activities. Unfortunately, long reaction times are required to achieve extensive protein modification (*21,22*).

PEG-phenylcarbonate derivatives with intermediate reactivity have also been evaluated (*23*). On reaction with amino groups, they liberate *p*-nitrophenol and 2,4,5-trichlorophenol, which are toxic and exhibit high protein affinity.

The succinimidyl succinate derivative of PEG reacts readily with proteins over a short period of time under mild conditions (30 min, pH ~ 7.5, 25°C) producing extensively modified proteins with well-preserved biological activities (*24,25*). The ester linkage between the polymer and the succinic ester residue has, however, limited stability in aqueous media (*26,27*). Substitution of the succinate moiety by glutarate improves the resistance to hydrolysis of the ester linkage (*28*).

Finally, mention should be made of the *N*-hydroxysuccinimide ester of carboxymethylated PEG, which is one of the most powerful *N*-acylating PEG derivatives evaluated so far (*29,30*). Its high reactivity has two major disadvantages. It is less selective toward amino groups, and it must be synthesized within a very short period of time before being used. In addition to the above-quoted individual drawbacks, most of these PEG reagents shared in common the disadvantage of being rapidly hydrolyzed. Therefore, the observation that SC-PEG was less susceptible to hydrolysis while being highly reactive toward amino groups (*31*) prompted us to pursue the evaluation of this new PEG reagent. For that purpose, two hemoproteins, namely catalase and lactoperoxidase, and two other enzymes, lysozyme and papaya proteinase III, were subjected to modification with SC-PEG.

MATERIAL AND METHODS

General

Aldrich provided monomethoxy-PEG (mPEG) of mol wt 5 kDa, 4-dimethylamino-pyridine, *N*-hydroxysuccinimide (HOSu), fluorescamine, dithiothreitol, methylmethanethiolsulfonate, acetonitrile (HPLC grade), hydrogen peroxide and *N*-acetyl-L-tryptophan ethyl ester. *Micrococcus luteus*, beef liver catalase (type C40), bovine serum albumin (BSA), azocoll, and *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) were purchased

from Sigma Chemical Co. Phosgene (20% in toluene), *N,N'*-disuccinimidyl carbonate, and Brij 3S were provided by Fluka. Succinic anhydride and molecular sieves (3A, 4–8 mesh) were from Janssens Chimica. Boehinger Mannheim GmbH provided 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic) acid (ABTS), and Bachem, benzoyloxycarbonyl-phenylalanyl-L-arginyl-7-(4-methyl)coumarylamide (Z-Phe-Arg-NH-Mec).

Hen egg white lysozyme was obtained from BELOVO (Bastogne, Belgium) and lactoperoxidase from SODELAC (Recogne, Belgium). Lysozyme, catalase, and lactoperoxidase were used without further purification.

Papaya proteinase III was purified from spray-dried papain generously given by ENZYMASE INTERNATIONAL S.A. (Brussels, Belgium). Spray-dried papain (2 g) was dissolved in 5 m*M* dithiothreitol and dialyzed, at 4°C for 20 h, against 1 m*M* EDTA at pH 5.0. Methylmethanethiolsulfonate was then added to the proteinase solution until the amidase activity against BAPA was abolished. The resulting enzyme solution was then fractionated on a CM-Sephadex C-50 column as described previously (*32*). Succinylated enzymes were prepared according to a procedure adapted from ref. (*33*).

Cyclohexyl-*N*-succinimide carbonate (CSC) was synthesized as described previously (*34*). Starting with *N,N'*-disuccinimidyl carbonate (1.25 mmol), cyclohexanol (1.25 mmol), and 4-dimethylamino-pyridine (0.625 mmol), CSC was obtained with a yield of 62% (mp: 94–95°C). I.R. (film on KBr, cm^{-1}) characteristic bands at 1818 and 1787 (both C =O, succinimide) and 1733 (C = O, carbonate). ^{1}H-NMR ($CDCl_3$) (δ in ppm): 1.21–2.05 (10 H, aliphatic multiplet), 2.83 (4 H, singulet,—CH_2CH_2—succinimido ring), and 4.76 (1 H, heptuplet, CH—O).

SC-PEG was synthesized from PEG chloroformate and HOSu as described previously (*31*). Dried organic solvents were stored in the presence of molecular sieves.

Enzyme Assays

The bacteriolytic activity of lysozyme was measured by using a suspension of *Micrococcus luteus* (*35*). One unit of activity is the amount of enzyme that produced a ΔA_{450} of 0.001/min at pH 6.24 (66 m*M* phosphate buffer containing 150 m*M* NaCl) and 25°C.

Papaya proteinase III was assayed using BAPA as the substrate as described previously (*32*). This proteinase was also assayed using Z-Phe-Arg-NH-Mec as the substrate. In this case, the buffer contained 100 m*M* phosphate, 1 m*M* EDTA, 2 m*M* dithiothreitol, and 0.005% Brij 35 at pH 6.80. Each test vessel (total vol: 3 mL) contained 50 μM of substrate, 12 m*M* of enzyme, and 6% methanol (MeOH). Stock solutions of substrate were prepared in MeOH. The enzyme was activated, at 40°C, in 750 μL of fourfold concentrated buffer in a fluorimeter cuvet for 2 min. Prewarmed (40°C) water was then added followed by substrate. The liberation of

7-amino-4-methyl-coumarin was measured fluorimetrically (excitation: 360 nm; emission: 460 nm).

The activity of lactoperoxidase (0.05 mg/mL; 5 μL) was measured using H_2O_2 (0.1 m*M*; 13 μL) and ABTS (0.5 m*M*; 2 mL) as substrates. Solutions were prepared in a buffer that contained phosphate, borate, and citrate, 25 m*M* each, at pH 5.50. The amount of oxidized ABTS was recorded spectrometrically at 436 nm (using ϵ_{436} for ABTS = $29300 M^{-1}.cm^{-1}$). One unit of lactoperoxidase is the amount of enzyme that oxidized 1 μmol of ABTS/min at 25°C.

Catalase was assayed with H_2O_2 as the substrate. An enzyme solution containing approx 50 U/mL (100 μL) was added to 2.9 mL of H_2O_2 (12–12.5 m*M*) in 50 m*M* phosphate at pH 7.0. The time required for A_{240} to decrease from 0.450 to 0.400 was measured. The amount of H_2O_2 that had disappeared was calculated using ϵ_{240} for H_2O_2 = 43.6 $M^{-1}.cm^{-1}$ (*17*). One unit of catalase is the amount of enzyme that decomposed 1 μmol of H_2O_2/min at 25°C. Stock solutions (10 mg/mL) of catalase were prepared in 50 m*M* phosphate buffer at pH 7.0 containing 30% glycerol and 10% EtOH, and kept at 4°C.

SDS-PAGE

The migrations were performed on slab gels using the mini protean II cell (Bio-Rad). The resolving gels (pH 8.8; 15% acrylamide) were run at a constant voltage (200 V) and prepared according to the method of Laemmli (*36*). The stacking gels were 4% polyacrylamide (pH 6.8). The upper and lower chambers contained Tris-glycine buffer (pH 8.3) with 0.1% SDS. The separation was toward the anode, and bromophenol blue was used as the tracking dye. Prior to loading on the gel, the protein solutions were diluted with buffer (buffer composition: 0.25*M* Tris-HCl at pH 6.8; 4% SDS; 23% glycerol; 1% bromophenol blue, and 5 m*M* dithiothreitol) and boiled for 4 min. The gels were stained with 0.25% Coomassie Blue R250 dissolved in the washing solution. The latter was also used for destaining, and contained MeOH, HOAC, and H_2O (45/10/45; v/v).

Fourier Transform Infrared Spectroscopy

IR spectra were recorded with a Perkin-Elmer Infrared Spectrometer 1720X equipped with a Perkin-Elmer microspecular reflectance accessory (ref. P.E. 221-0357) (*37*). The internal reflection element was a Germanium ATR plate (50 × 20 × 2 mm. Harrick EJ 2121) with an aperture angle of 45° yielding about 25 internal reflections. The spectrophotometer was continuously purged with air dried on a silica column (5 × 130 cm) at a flow rate of 7 L/min. Spectra were recorded with a nominal resolution of 2 cm^{-1} and, after scanning, were transferred to a computer.

Other Spectroscopic Measurements

^{1}H-NMR spectra were obtained with a Brucker WP 250 Fourier transform spectrometer operating at 250.13 MHz. Chemical shifts were expressed

in ppm relative to TMS as the internal reference. The infrared spectra of organic compounds were obtained with a Shimadzu IR-470 spectrophotometer. Absorption spectra were achieved with a Cary model 118 spectrophotometer, and fluorescence spectra with a Shimadzu RF-5100-PC spectrofluorimeter.

Analytical Methods

The protein concentrations were determined spectrophotometrically using A1% values of 28.5 at 282 nm for lysozyme, 18.3 at 280 nm for papaya proteinase III, 14.7 at 412 nm for lactoperoxidase, and 6.01 at 405 nm for catalase. Free amino functions of proteins were determined fluorimetrically after reaction with fluorescamine (*38*). Automatic titrations were performed on a Radiometer pH-stat made up of a PHM 84-meter coupled with a TTT80 titrator, an ABU 80 autoburette, and a REC 80 servograph recorder.

RESULTS AND DISCUSSION

The optimal pH for use of the new reagent SC-PEG has been reported to be ca. 9.3. This was suggested by the pH dependence of the ratio of the rates of aminolysis vs hydrolysis exhibited by SC-PEG (*31*). Not all enzymes, unfortunately, could withstand such conditions, even for a short time. As a consequence, we found it advisable to evaluate the performances of this new *N*-acylating reagent at pH 7.0, too. Four enzymes, belonging to different families, were chosen for this purpose. Three among of them (papaya proteinase III, lysozyme, and lactoperoxidase) are monomeric enzymes, whereas catalase is tetrameric.

Rates of *N*-Acylation with SC-PEG

A number of modifications were first carried out using different molar ratios of SC-PEG to papaya proteinase III at pH 7.0 and 25°C. Ten milliliters of an aqueous solution of the protein (5 mg/mL) were adjusted to pH 7 by automatic titration with 10 m*M* NaOH. Solid SC-PEG was added under stirring, and titration started with the same NaOH solution. The reaction was allowed to proceed until a plateau value was reached (about 140 min). The sample was then submitted to extensive dialysis and analyzed. The protein content was determined spectrophotometrically, whereas the residual content of primary amino functions was determined by a fluorimetric assay as shown in Fig. 1. This figure also displays the results for native and fully succinylated papaya proteinase III. The native proteinase, which contains 23 mol of NH_2/mol of protein, was taken as 100%, and the succinylated derivative as 0% (*39*). The percentages of unreacted lysyl residues were calculated from the slopes of the curves of Fig. 1.

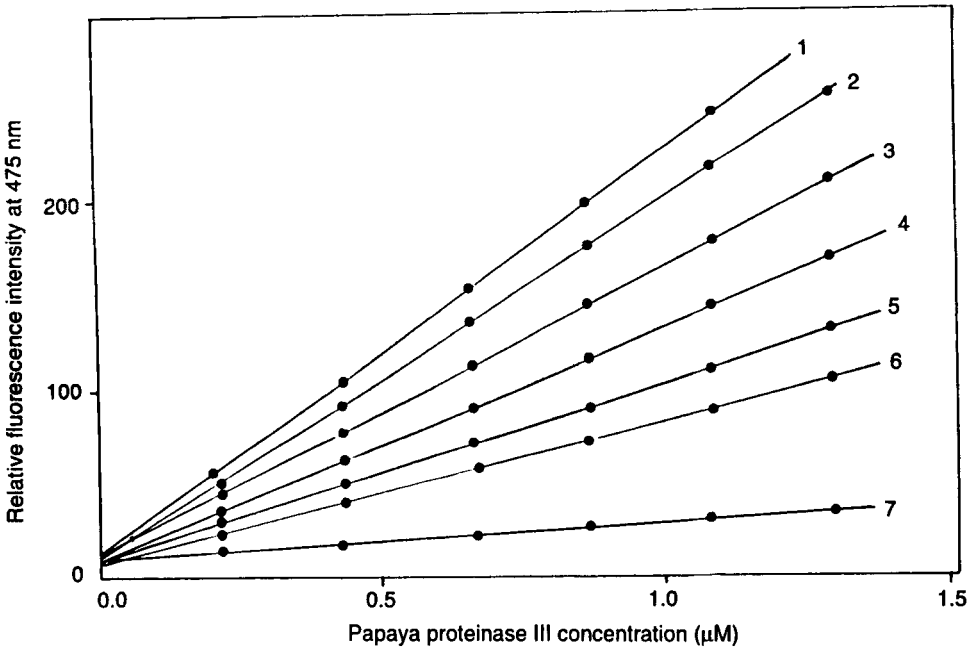

Fig. 1. Fluorimetric assay of free amino functions of papaya proteinase III. Curves 1 and 7 were obtained with the native and the succinylated proteinase, respectively. Curves 2–6 were obtained with the proteinase after reaction with SC-PEG using 0.25, 0.50, 1.00, 3.00, and 4.00 mol, respectively, of SC-PEG/mol of NH_2 from the enzyme. For the fluorimetric assay, the protein material was diluted in 100 m*M* phosphate at pH 8.0. Fluorescamine (0.3 mg/mL) in acetone (0.5 mL) was added to 1.5 mL of the diluted solution while vortexing. After a minimum of 10 min, the solutions' emission spectrum was recorded (excitation at 390 nm).

One can see in Fig. 2 that if low ratios of reagent to amino group (2 to 5) are used, papaya proteinase III may be acylated to a great extent (16 sites out of 23 theoretically modifiable) even at pH 7.0. In the case of hen egg white lysozyme, 6 PEG chains (out of the seven possible ones) were linked by using 2 mol of SC-PEG/mol of NH_2 (*see* Fig. 3).

We also intended to acylate two hemoproteins, i.e., catalase and lactoperoxidase. Unfortunately, the degree of linking of mPEG to these enzymes could not be determined by the fluorescamine assay owing to the interference of the 405-nm absorption band of these hemoproteins. As a consequence, we arbitarily chose an SC-PEG to NH_2 molar ratio susceptible to produce an exhaustively modified conjugate, i.e., 2 for catalase and 3 for lactoperoxidase (plateau value for the other proteins tested).

SDS-PAGE experiments showed that acylation of catalase and lactoperoxidase did actually occur to a great extent. In marked contrast to the native (unmodified) enzymes (*see* Fig. 4), that migrated as single

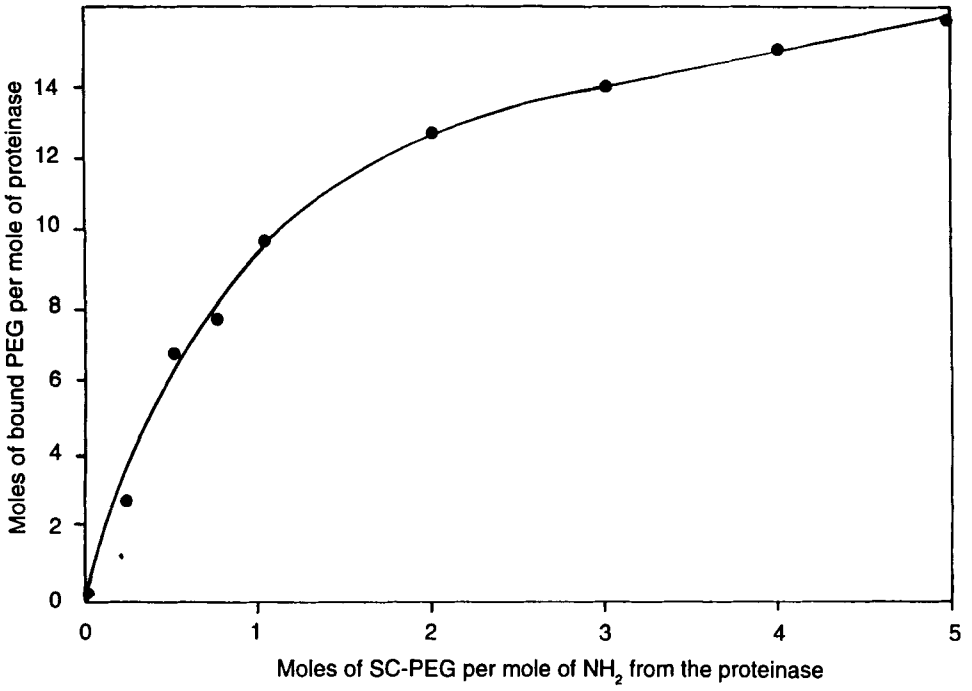

Fig. 2. Titration curve of papaya proteinase III by SC-PEG. Each dot represents the results of one separate experiment. Modifications with SC-PEG were carried out at pH 7.0 and 25°C. At the end of the reactions, the reaction mixtures were dialyzed (15 h, 4°C, 3 × 5 L H_2O) and analyzed (protein content, residual content of amino functions).

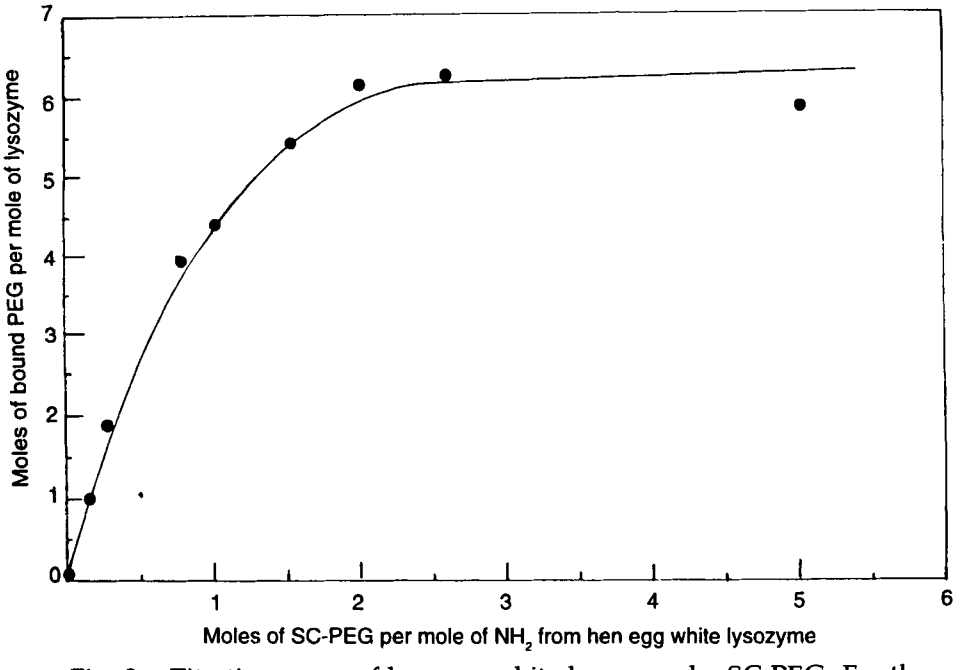

Fig. 3. Titration curve of hen egg white lysozyme by SC-PEG. For the experimental conditions, *see* Fig. 2.

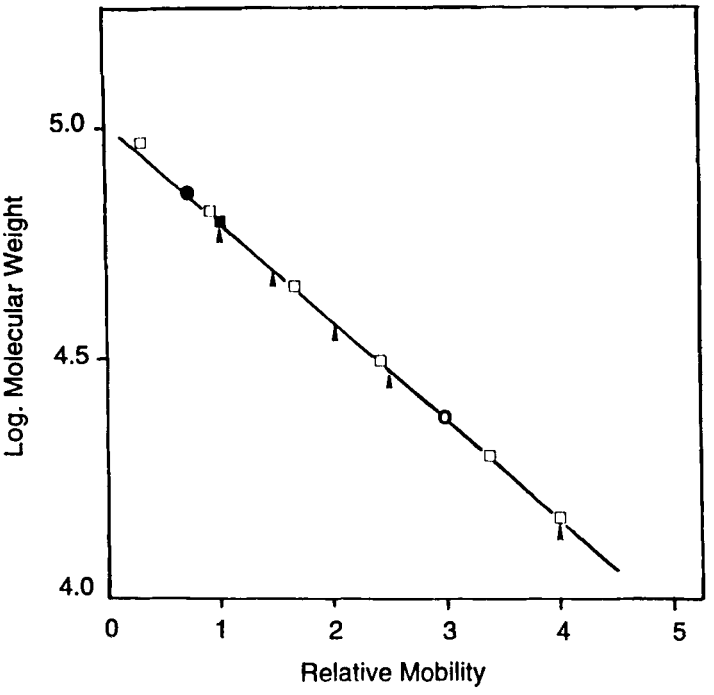

Fig. 4. Semilogarithmic plot of molecular weight vs migration distance for papaya proteinase III (○), catalase (■), lactoperoxydase (●), and the protein markers (□). The *Mr* markers were lysozyme (14.3 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase B (97.4 kDa). For further details, *see* under the experimental section. Arrows indicate the relative mobility of the various bands found after the reaction of hen egg white lysozyme with three equivalents of SC-PEG.

bands with molecular weight of 74,130 (lactoperoxidase) and 61,660 dalton (catalase), their mPEG conjugates did not penetrate into the gels. Moreover, no trace of the original enzymes or of species of intermediate molecular weight appeared.

When a less exhaustive modification was attempted, such intermediates were visible on the SDS-PAGE gels. This is the case for example for lysozyme after modification with 3 mol of SC-PEG/mol of enzyme (*see* Fig. 4), which leads to a complex mixture containing five different molecular species of different molecular weights.

Taking into account the results reported by Zalipsky and coworkers (*31*) and those shown here, one may conclude that SC-PEG is truly the reagent of choice to perform exhaustive modifications of proteins with PEG chains under physiological, nondenaturing conditions for most enzymes. Such exhaustive modifications, which are required for many applications (*1–7,15,16*) could generally not be achieved previously without a substantial loss of biological activity (*39,40*).

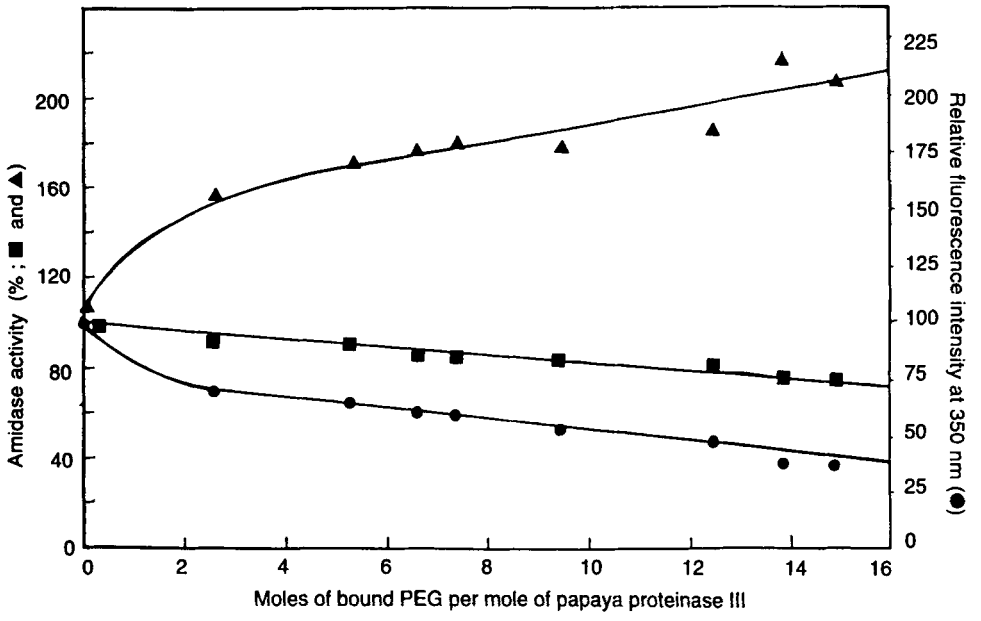

Fig. 5. Effect of increasing rates of acylation of papaya proteinase on the amidase activity and intrinsic fluorescence emission (●). Amidase activities were measured using BAPA (■) and Z-Phe-Arg-NH-Mec (▲) as described in the experimental section. Fluorescence emission spectra were recorded at 25°C (excitation at 285 nm) at pH 5.0.

Effect of the Modification with SC-PEG on the Enzymic Activity

The different PEG–lysozyme conjugates that were assayed contained between 1.00 and 6.19 mol of covalently bound PEG/mol of enzyme. All these PEG-modified lysozyme derivatives lacked (<1% of native) bacteriological activity against *Micrococcus luteus*.

When macromolecular substrates were used, acylated papaya proteinase III also showed a lack of proteolytic activity. PEG–proteinase conjugates containing between 6.4 and 15.8 mol of bound PEG/mol of enzyme were completely inactive toward azocoll (*41*) and bovine serum albumin. In the latter case, no fragment could indeed be observed by SDS-PAGE.

In marked contrast, as shown in Fig. 5, the amidase activity remained well preserved against BAPA and even enhanced against Z-Phe-Arg-NH-Mec. On acylation with SC-PEG, catalase also showed a dramatic increase in its specific activity from 8500 U/mg protein (native enzyme) up to 13,000 U/mg protein (acylated enzyme). The absorption spectrum of catalase in the UV and visible ranges was, however, unaffected by acylation. Finally, lactoperoxidase assay showed 167–168 U/mg protein both before and after PEG conjugation.

Acylation by PEG of proteins that act or interact on macromolecular substrates often resulted in a complete loss of activity. This observation

has been reported on numerous enzymes, such as trypsin (*31*), ribonuclease (*42*), or sphingomyelinase (*43*). Nevertheless, the same PEG–enzyme conjugates retained their activity against small substrates, thus outlining the important role of the steric repulsions exerted by PEG on hydrophilic macromolecular substrates. This feature was observed here when assaying the bacteriolytic and proteolytic activities of acylated lysozyme and acylated papaya proteinase III, respectively.

On the other hand, enhancement of the specific activity of the PEG–enzyme conjugates over the native enzymes was observed in two circumstances. Such an observation has been rarely reported in the literature. An increase in α2-macroglobulin-trypsin amidolytic activity has been shown to result from PEG attachment (*21*). PEG-modified trypsin also showed enhanced activity toward nitroanilide substrates (*31*). Conjugation of PEG to β-galactosidase similarly resulted in increased activity toward hydrophobic substrates and in substantial activity toward GM1-ganglioside, which cannot be hydrolyzed by the native enzyme in the absence of detergents (*44*). Finally, it should be mentioned that the coupling of a copolymer of ethylene and maleic anhydride to alkaline phosphatase increases its enzymatic activity 26-fold at pH 7.1, and 10-fold at pH 8.6 compared with the unmodified enzyme (*45*).

At the present time, no clear explanation has yet been offered of the cause of such activity enhancements. Nonetheless, the increased activities measured here may be interestingly used for the comparison of SC-PEG with other PEG coupling agents. Modification of 70% of the amino groups of catalase using PEG activated by cyanuric chloride led, in the best case, to 100% activity (*17*), whereas 153% was observed here. As far as we know, lactoperoxidase has not previously been modified with PEG. It may, however, be compared to horseradish peroxidase (HRP), which acts on the same substrates H_2O_2 and ABTS. Conjugation of PEG to 60% of the available lysyl residues of HRP led to an enzyme preparation that possessed a residual activity of 70% of the starting enzyme preparation (*3*).

Papaya proteinase III, on the other hand, may be compared to papain, since their amino acid sequences showed 80% homology (*46*). Papain has been previously conjugated with PEG activated with cyanuric chloride, resulting in modification of 76% of the amino functions (*2*). When assayed against the synthetic substrate Pyr-Phe-Leu-*p*-nitroanilide, only 30% of the initial activity was recovered (*2*). Thus, quite obviously, SC-PEG offers the best recoveries of enzymatic activity when used as a coupling reagent for enzyme acylation under conditions such as those recommended here.

Effect of the Acylation with SC-PEG on Protein Folding

The high specific activities that characterize most of the PEG–enzyme conjugates prepared here from SC-PEG might lead one to expect that the 3-D structures of the enzymes are rather unaffected by the covalent

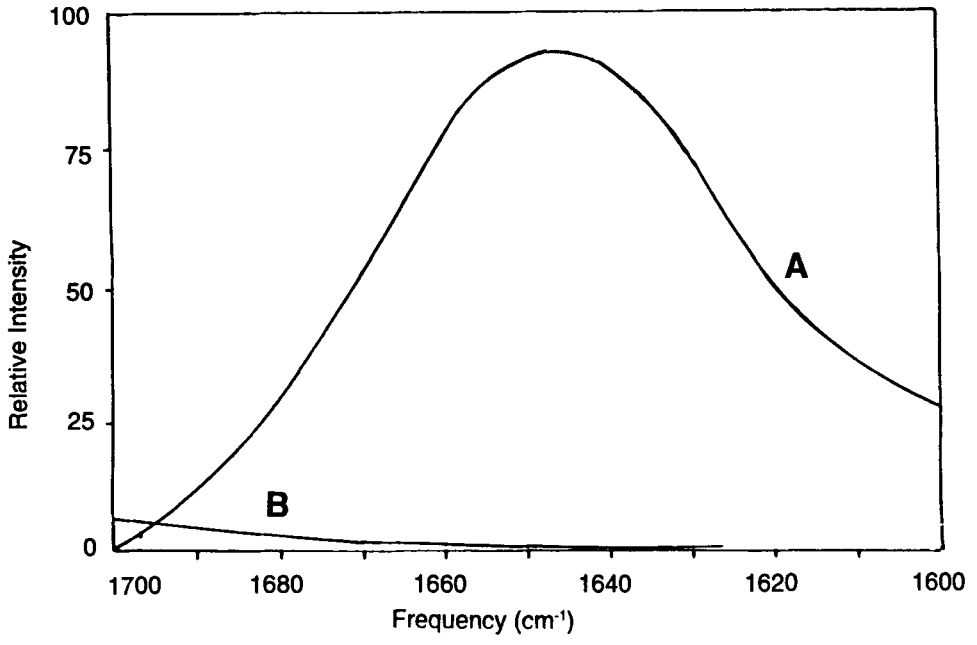

Fig. 6. Infrared spectra in the amide I′ region of lactoperoxidase (A) and mPEG—O—C—NH—CH_2—CH_2—$NH_3^+Cl^-$ (B); 30 mg each at pH 5.0.
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attachment of PEG chains. Such an inference has already been reported several times in the literature (*42,47-49*). However, in the course of this work, it was observed that lactoperoxidase was the sole enzyme whose specific activity remained strictly unchanged after PEG attachment.

Some physicochemical characterizations, thus, were carried out to examine whether structural changes do occur on PEG modification, and do or do not contribute to alteration of enzymatic activity. Fourier transform infrared spectroscopy (FTIR) was used to determine whether conjugation of PEG chains alters the enzymes' secondary structures. The determination of the proportions of their different elements was carried out as described previously (*50*). We used the frequency range of 1662-1645 cm^{-1} for α-helix, 1689-1682 and 1637-1613 cm^{-1} for β-sheet, 1682-1662.5 cm^{-1} for β-turns, and 1644.5-1637 cm^{-1} for the aperiodic structures.

This 1600-1700 cm^{-1} range, which was explored, corresponds to the amide I′ region. Conjugation of PEG to proteins using SC-PEG introduces carbamate functions. It was thus of prime interest to check whether these carbamate functions, present in the PEG-enzyme conjugates, do not interfere. Figure 6 displays the IR spectrum of native lactoperoxidase together with that of mPEG—O—C—NH—CH_2—CH_2—$NH_3^+Cl^-$ (22).
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Table 1
Compilation of the Secondary Structure Contents of Lysozyme, Papaya Proteinase III, Catalase, and Lactoperoxidase and Their PEG Conjugates

Enzyme	Mol of-SC-PEG/mol of NH_2	Percent			
		α-Helix	β-sheet	β-turns	aperiodic
Native lysozyme		32.7	26.9	23.9	16.4
PEG-lysozyme	5	39.5	21.5	23.6	15.4
Native papaya proteinase[1]		26.4	39.5	25.9	8.2
PEG-papaya proteinase[1]	5	28.6	37.1	22.0	12.3
Native catalase		26.7	38.2	20.2	14.9
PEG-catalase	2	33.5	29.1	23.0	14.3
Native lactoperoxidase		27.8	27.6	29.4	15.2
PEG-lactoperoxidase	3	29.1	37.5	21.0	12.5

[1]As the *S*-methylthioderivative.

Examination of Fig. 6 reveals that indeed the carbamate function is quite transparent in the frequency range of interest. As a consequence, FTIR may be used to probe the secondary structure of PEG–protein adducts wherein the PEG chains are linked through carbamate functions. The results of the FTIR study are shown in Table 1. The secondary structures of the four enzymes were compared with the secondary structures of their PEG-adducts. These adducts were obtained by using ratios of 2 or 3 SC-PEG/mol of amino function to yield exhaustively acylated enzymes.

Examination of the results in Table 1 reveals that the helical contents were higher in the conjugates than in the native enzymes. Moreover, with the exception of lactoperoxidase, modification of the proteins with PEG reduced their β-sheet contents. It should finally be observed that the β-turn structures (with the exception, again, of lactoperoxidase) and the aperiodic sequences were weakly affected by the PEG-adduct formations.

However, taking into consideration the limits of the reliability of the method (±5%), one should not distinguish lysozyme from PEG-lysozyme or papaya proteinase from the PEG papaya proteinase conjugate. Significant differences were observed only in the case of the two hemoproteins catalase and lactoperoxidase. Other spectroscopic techniques, such as absorption and fluorescence, that have been often selected to evaluate structural changes in PEG-protein adducts, were also used here.

We observed that the UV and the visible absorption spectra of both PEG-lactoperoxidase and PEG-catalase were quite superimposable on the spectra of the native enzymes. Also, the fluorescence emission spectra (λ excitation: 285 nm, pH 5, 25°C) of the different PEG–lysozyme conjugates were identical to those of the native proteins.

In marked contrast, as shown in Fig. 5, the fluorescence of papaya proteinase III decreased as a function of the acylation rate of the proteinase. When 16 chains of PEG were covalently bound to papaya proteinase III, the residual fluorescence was only 40% of the value of the native protein. This residual fluorescence was also characterized by a shift of λ_{max} toward shorter wavelengths. Qualitatively and quantitatively, the quenching that resulted from the PEG conjugation closely resembles the quenching of Trp-177 of the proteinase (*51*). This residue is quite near the active site of the proteinase. In agreement with this interpretation, one can observe from Fig. 5 that the decrease in the fluorescence on incorporation of PEG chains in the proteinase parallels the increase in amidase activity (substrate: Z-Phe-Arg-NH-Mec). Thus, it is expected that fluorescence, in the case of papaya proteinase III, may not be a suitable parameter to evaluate the effect of covalently bound PEG chains on the overall structure of the enzyme.

In summary, we have not been able to put forward significant differences between the folding patterns of the PEG–protein conjugates and the native enzymes. This conclusion holds true for lysozyme, which lost its bacteriolytic activity against *Micrococcus luteus* after the covalent attachment of PEG. Thus, the substantial rates of acylation achieved with SC-PEG do not constitute a handicap *per se*.

Finally, the synthesis of SC-PEG, as described by Zalipsky and coworkers (*31*), is the sole apparent drawback of this reagent. Its synthesis made use of phosgene, which could indeed bridle the desires of those less skilled in the art of organic synthesis. Thereafter, we thus sought a novel route for the synthesis of SC-PEG.

Synthesis of SC-PEG from *N,N'*-Disuccinimidyl Carbonate

During the synthesis of CSC, no evidence of dicyclohexyl carbonate formation appeared. This encouraged us to attempt the synthesis of SC-mPEG using the same schematic route.

The following experimental protocol proved to be the most suitable: mPEG and 4-dimethylaminopyridine, 5 mmol each, were dissolved in toluene (250 mL). Traces of water present in mPEG (0,4% according to the Karl Fischer titration) were removed by azeotropic distillation of 100 mL of toluene. After cooling (25°C) *N,N'*-disuccinimidyl carbonate (5.5 mmol) was added to the mPEG solution. The reaction proceeded for 50 h at room temperature in a moisture-free atmosphere. The *N*-hydroxysuccinimide formed in the course of the reaction was then eliminated by filtration, and the clear filtrate added dropwise to 850 mL of diethyl ether under vigorous stirring. SC-PEG was collected by filtration, washed thoroughly with ether, and dried under vacuum in the presence of P_2O_5. The formation of SC-PEG was confirmed by the Infrared Spectrum, which displayed the three expected characteristic bands at 1741, 1788,

and 1805 cm^{-1} attributed to the C=O vibrations. No new band resulting from the presence of di-PEG carbonate (unpublished results) could be detected in the 1700–1850 cm^{-1} range where mPEG is known to be perfectly transparent.

Twenty-five grams of SC-PEG preparation were obtained. However, unreacted mPEG and SC-PEG could not be separated from each other. For this reason, the yield of mPEG derivatives could not be determined, as is calculated conventionally, from the weight of collected synthesized material. Spectrophotometrical techniques were indeed unsuitable since CSC and SC-PEG do not show any characteristic spectral band in the near-UV range where mPEG is transparent.

On hydrolysis, SC-PEG converts into mPEG, CO_2, and *N*-hydroxysuccinimide. The salts of the latter display an UV absorption band with a maximum at 259 nm, an ϵ_{259} value of 8700 $M^{-1}.cm^{-1}$, and an apparent pK_a of 5.86 (determined spectrophotometrically).

The spectrophotometric determination of N-hydroxysuccinimide formed on hydrolysis of SC-PEG gave us a first measurement of the yield of this PEG derivative. On the other hand, protons released during the hydrolysis of SC-PEG could be titrated automatically, a second way to calculate the yield of SC-PEG. Practically, the spectrophotometric determinations of *N*-hydroxysuccinimide were performed on 4-h-old solutions of SC-PEG (0.05–0.1 m*M*) in 100 m*M* Tris-HCl buffer at pH 8.50, whereas on the other hand, aqueous solutions of SC-PEG (5.0 g; 1 m*M*) in 50 m*M* $NaHCO_3$–Na_2CO_3 buffer at pH 10.25 were prepared immediately before automatic titration. pH was maintained at 10.25 by adding 10 m*M* NaOH. Under these experimental conditions, a ratio of 2.5 mol NaOH/mol SC-PEG was used to calculate the number of mol of hydrolyzed SC-PEG (hydrolysis at pH 10.25 reached completion within 30 min). The two different methods gave identical results and allowed us to conclude that the SC-PEG derivative constituted 80% of the preparation.

CONCLUDING REMARKS

SC-mPEG offers many advantages as compared to other mPEG derivatives used to introduce PEG chains into polypeptides. Some of these advantages, evaluated using trypsin as the protein model, were already outlined in the publication of Zalipsky and coworkers (*31*). They are largely confirmed by the results reported here, which were obtained using four additional enzymes, namely: lysozyme, papaya proteinase III, lactoperoxidase, and catalase.

Two routes are now available for the obtention of SC-mPEG in high yield (80–85%). The first one, described by Zalipsky and coworkers (*31*), uses phosgene and *N*-hydroxysuccinimide. Both chemicals are inexpensive, which makes this method particularly attractive in the perspective of

industrial applications. In the second approach reported here, the engaged reagents are *N,N'*-disuccinimidyl carbonate and 4-dimethylaminopyridine. This method might be preferred by those working at laboratory scale, since it makes use of reagents that may be qualified of safe.

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