

A New Procedure for the Synthesis of Polyethylene Glycol-Protein Adducts; Effects on Function, Receptor Recognition, and Clearance of Superoxide Dismutase, Lactoferrin, and α_2 -Macroglobulin

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A new, simplified technique for the synthesis of polyethylene glycol (PEG) derivatives of proteins utilizing 1,1'-carbonyldiimidazole for PEG activation, is described. PEG derivatives of superoxide dismutase, α_2 -macroglobulin, α_2 -macroglobulin-trypsin, and lactoferrin were prepared and studied. Superoxide dismutase coupled to PEG preserved 95% of its original activity while its plasma half-life increased from 3.5 min to 9 or more hours depending on the PEG derivative studied. PEG-derivatized α_2 -macroglobulin showed decreased protease binding activity but PEG derivatives of preformed α_2 -macroglobulin-trypsin demonstrated no loss of activity. The plasma clearance of PEG- α_2 -macroglobulin-trypsin was prolonged significantly compared to native α_2 -macroglobulin-trypsin, particularly when a high-molecular-weight PEG was coupled to the protease inhibitor complex. The plasma clearance half-life of lactoferrin was increased 5- to 20-fold by this modification. Trinitrobenzenesulfonic acid titration studies demonstrated that ϵ -amino groups of lysine residues are modified by the coupling of carbonyldiimidazole-activated PEG to proteins.

KEY WORDS: polyethylene glycol-modified proteins; superoxide dismutase; lactoferrin; α_2 -macroglobulin.

The hydroxyl groups of monomethoxy-polyethylene glycols (HO(-CH₂-CH₂-O-)_nCH₃) may be activated by cyanuric chloride and several other reagents and then coupled to lysine ϵ -amino groups of proteins (1-5). Polyethylene glycol-protein adducts demonstrate increased blood circulation time and decreased antigenicity and immunogenicity as compared to the unmodified proteins (1,6-12). Derivatized proteins, however, often demonstrate a marked loss of activity (8,10).

1,1'-Carbonyldiimidazole reacts with the hydroxyl groups on agarose to produce a derivative that reacts with nucleophiles (13). In the present manuscript a simple procedure is described for activating polyethylene glycol with this reagent. Reactions with monofunc-

tional (PEG-2 and PEG-5),² bifunctional (PEG-4), and tetrafunctional (PEG-20) polymers containing one, two, and four hydroxyl groups, respectively, are compared.³ Polyethylene glycols activated with this reagent couple to lysine residues as with other activated polyethylene glycols.

² Abbreviations used: PEG, polyethylene glycol; SOD, superoxide dismutase; α_2 M, α_2 -macroglobulin; BAPNA, N^α-benzoyl-DL-arginine-*p*-nitroanilide-HCl; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

³ The monofunctional polyethylene glycols, PEG-2 and PEG-5, each have one of their hydroxyl groups replaced by a methoxy group. The bifunctional polyethylene glycol, PEG-4, has two free hydroxyl groups. PEG-20, as synthesized by the manufacturer has four free hydroxyl groups (Sigma Chemical Co., Technical Services Division). The number designations for PEG species indicate the average molecular weight of the product.

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The proteins derivatized with the activated polyethylene glycols include bovine liver copper-zinc superoxide dismutase, human plasma α_2 -macroglobulin complexed with trypsin, and human milk lactoferrin. These proteins are representative of three mechanisms of plasma clearance. Superoxide dismutase, $M_r = 31,200$, catalyzes the disproportionation of O_2^- (14). Clearance from the mammalian circulation results from passive diffusion of the relatively small protein through the renal glomeruli (15). Lactoferrin is an iron-binding protein found in the granules of neutrophils which may play an important role in tissue injury mediated by phagocytes (16). It is rapidly cleared from the circulation by hepatocytes as a result of carbohydrate recognition (17). α_2 -Macroglobulin is a high-molecular-weight plasma protease inhibitor (18). Following reaction with a protease, the complex is recognized by receptors in the reticuloendothelial system and rapid clearance occurs (19). The receptor recognition site is present on the inhibitor and most likely involves a sequence of amino acids which either becomes exposed or assumes an appropriate conformation after reaction with protease.

The clearance rates of the native and polyethylene glycol-derivatized forms of these proteins are compared in mice. The results of *in vitro* activity experiments are also presented. Using published data and these studies, a comparison is made of some of the advantages and disadvantages of the cyanuric chloride and 1,1'-carbonyldiimidazole procedures for synthesis and polyethylene glycol-protein derivatives.

MATERIALS AND METHODS

Materials. Bovine serum albumin, trypsin, BAPNA, 1,1'-carbonyldiimidazole, TNBS, *p*-nitrophenyl-*p*'-guanidinobenzoate-HCl, cytochrome *c* type IV and SBTI were obtained from Sigma. Trypsin, purchased from Worthington, was 70% active by active-site titra-

tion with *p*-nitrophenyl-*p*'-guanidinobenzoate-HCl (20). Burdick-Jackson ultrapure dioxane was obtained from Anspec, Ann Arbor, Michigan; lactoperoxidase bound to Sepharose-4B from P-L Biochemicals; ^{125}I for protein iodination from New England Nuclear; DE-52 from Whatman; electrophoresis reagents from Bio-Rad; and ultrapure ammonium sulfate from Schwartz/Mann. Spectrapor dialysis tubing was obtained from Fisher. PEG-4 (Carbowax 4000) was purchased from Fisher, PEG-2 and PEG-5 from Polyscience, and PEG-20 from Sigma. Lactoferrin prepared according to Querinjean *et al.* (21) was generously supplied by Jean-Paul Prieels, Universite Libre de Bruxelles. Human plasma α_2M was purified by a modification (19) of the method of Kurecki *et al.* (22). All other reagents were the best commercial grade available.

Electrophoresis. Native polyacrylamide gel electrophoresis was performed on 5% slabs with the continuous Tris-borate system of Nelles *et al.* (23) or on 7.5% gels using the discontinuous buffer system of Davis (24). Gels were stained with Coomassie brilliant blue G-250.

Enzyme activity assays. SOD activity was measured according to the procedure of McCord and Fridovich (14). The ability of α_2M to bind trypsin following reaction with PEG was studied with a modification of the method of Ganrot (28). A detailed description of the procedure is presented elsewhere (29). In other experiments, α_2M -trypsin complex was preformed and then reacted with activated PEG. BAPNA substrate was added and amidolytic activity was assayed.

Protein concentrations. The concentrations of unmodified proteins were calculated using the following extinction coefficients and molecular weight determinations: α_2M , $A_1^{1\% \text{ cm. } 280 \text{ nm}} = 8.93$, $M_r = 718,000$ (25), lactoferrin, $A_1^{1\% \text{ cm. } 280 \text{ nm}} = 10$, $M_r = 77,000$ (21), bovine copper-zinc SOD, $A_1^{1\% \text{ cm. } 280 \text{ nm}} = 2.70$, $M_r = 31,200$ (14,26). The concentration of PEG-modified protein was estimated by the

Biuret procedure (27). The unmodified proteins served as standards.

Purification of bovine liver copper-zinc SOD. The procedure of McCord and Fridovich (14) for isolating SOD from bovine erythrocytes was modified for a bovine liver source. All steps were performed at 4°C unless otherwise specified. Fresh bovine liver (900 g) was homogenized in a Waring blender for 2 min in 2 liters of deionized water and then filtered through cheese cloth. Ethanol (0.25 vol) and chloroform (0.15 vol) were added with stirring and the mixture centrifuged (9200g) for 30 min. To the supernatant, 300 g/liter of anhydrous K_2HPO_4 was added with stirring and the preparation was centrifuged for 20 min at 21,000g. The supernatant was placed in a separatory funnel and the upper ethanol phase was collected and centrifuged for 20 min at 21,000g. The supernatant was again placed in a separatory funnel to remove and discard residual lower phase. The upper ethanol phase containing SOD activity was rapidly cooled to between -2 and -5°C in a salt-ice bath and 0.75 vol of acetone at -20°C were added with stirring. The mixture was immediately centrifuged (21,000g) for 20 min and the precipitate collected and dispersed with a Tenbroek homogenizer in 100 cm³ cold deionized H₂O. Ammonium sulfate (32.6 g/100 ml) was added, the mixture centrifuged (21,000g) for 20 min, and the precipitate discarded. The supernatant was dialyzed against 2.5 mM KP_i , pH 7.45 and applied to a DE-52 column (2.5 × 35 cm) equilibrated in the same buffer at 4°C. The column was eluted sequentially at pH 7.45 with 250 ml each of 2.5 mM KP_i , and 10 mM KP_i with 7 ml fractions collected at 2 ml/min. SOD eluted from the column at the 10 mM KP_i step. Purity of the fractions was determined by polyacrylamide gel electrophoresis. Fractions containing pure SOD were pooled. Fractions containing significant amounts of impure SOD were pooled and rechromatographed on the same DE-52 column. The presence of two to three SOD isoenzymes was verified by comparison of gels

stained for protein with gels stained for SOD activity (30). The purified SOD had a specific activity of 3200 units/mg using the activity assay of McCord and Fridovich (14) and a protein determination as described by Murphy and Kies (31).

Radioiodination of proteins. Proteins were radiolabeled by the solid state lactoperoxidase procedure of David and Reisfeld (32). The specific activities in cpm/μg of the proteins were SOD, 1.3×10^4 ; lactoferrin, 5.7×10^4 ; and α_2M , 5.5×10^5 .

Activation of PEG. The PEG was dissolved in dioxane at 37°C at a concentration of 50 mM (PEG-2, PEG-4, and PEG-5) or 5 mM (PEG-20) and 1,1'-carbonyldiimidazole added to a final concentration of 500 mM. The solution was incubated at 37°C for 2 h with stirring and then dialyzed extensively against H₂O at 4°C. The activated PEG-2, PEG-4, and PEG-5 solutions were dialyzed in Spectrapor dialysis membranes with 1000, 2000, and 3500 M_r inclusion limits, respectively, and the activated PEG-20 solution was dialyzed in a dialysis membrane with a 10,000 M_r inclusion limit. The solutions were then lyophilized and stored desiccated at 4°C. The resultant powder constituted "activated PEG."

Elemental analysis of activated PEG derivatives. Analysis for percent hydrogen, carbon, oxygen, and nitrogen content of the activated PEG derivatives was performed by Spang Microanalytical Laboratory (Eagle Harbor, Mich.).

TNBS titration studies. The percentage of SOD amino groups that reacted with the activated PEG was determined using the TNBS titration procedure described by Glazer *et al.* (33). Prior to the TNBS titration, the PEG-SOD adducts were dialyzed in tubing with a 50,000 M_r cutoff and concentrated in an Amicon ultrafiltration apparatus with an XM-50 membrane.

Coupling of activated PEG to proteins. Activated PEG-4 and 5 (90 mM) and radiolabeled SOD (5×10^{-5} M) were incubated in 10 mM sodium borate, pH 8.5, and 4°C for 48

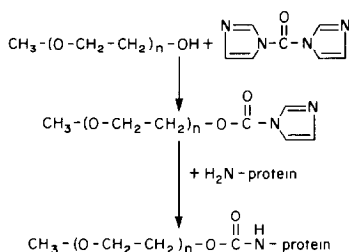


FIG. 1. Proposed mechanism for the formation of 1,1'-carbonyldiimidazole-activated monofunctional PEG and for the reaction of the activated PEG with amino groups of a protein.

h. At 48 h, the concentration of activated PEG-5 was increased to 180 mM and the incubation continued for another 48 h. In other studies, 180 mM PEG-5 was present for the entire 96-h incubation.

Trypsin was incubated with radiolabeled $\alpha_2\text{M}$ in 10 mM sodium borate, pH 8.5, for 5 min at room temperature at a trypsin to protease inhibitor ratio of 2. Activated PEG-2, PEG-4, and PEG-5 (40 mM) were incubated with 1×10^{-6} M $\alpha_2\text{M}$ -trypsin complex in the same buffer for 72 h. Activated PEG-20 (10 mM) was incubated with 5×10^{-7} M $\alpha_2\text{M}$ -trypsin complex for 72 h. Uncomplexed $\alpha_2\text{M}$ was reacted with PEG under similar conditions.

Activated PEG-2 or 4 (40 or 160 mM) and radiolabeled lactoferrin (4×10^{-6} M) were incubated in 10 mM sodium borate, pH 8.5, at 4 C° for 72 h.

Clearance studies. Clearance studies were performed in 20-week-old CD-1 female white mice as previously described (19,34). Studies were generally performed in triplicate and the results averaged. Each individual study varied by less than 5% compared to the mean.

RESULTS

Elemental analysis of activated PEG derivatives. The expected product of the reaction of 1,1'-carbonyldiimidazole with the hydroxyl group of PEG is an imidazole carbamate derivative (13). Figure 1 illustrates the proposed structure of activated PEG formed by this

procedure. The nitrogen content for each of the activated PEG derivatives was calculated using this general structure, and the predicted value compared to the actual content determined by elemental analysis of the activated PEG derivatives (Table 1). For each of the activated PEG derivatives, experimental nitrogen content was essentially identical to the calculated percent nitrogen from the structure of activated PEG in Fig. 1.

TNBS titration of the PEG derivatives of SOD. TNBS titration was performed to determine if amino groups of the PEG-derivatized protein were modified as predicted by the structure of the PEG-protein adduct given in Fig. 1. The PEG-4 and PEG-5 derivatives of SOD were extensively dialyzed in 50,000 M_r inclusion limits dialysis tubing and concentrated using a filtration membrane with a similar cutoff to remove PEG which interferes with TNBS titration (data not shown). The TNBS titration results showed the PEG-4 derivative of SOD had 5% and the PEG-5 derivative 10% titratable amino groups as compared to the unmodified protein.

Gel electrophoresis of PEG modified SOD. Polyacrylamide gel electrophoresis was used to demonstrate that incubation of activated PEG with SOD produces an altered form of the enzyme and to study the effect of different incubation conditions on the formation of a PEG-SOD adduct. The effect of two different

TABLE 1
ELEMENTAL ANALYSIS OF ACTIVATED
PEG DERIVATIVES

Activated PEG	% Nitrogen	
	Predicted	Found
PEG-2	1.3	1.1
PEG-4	1.6	1.6
PEG-5	0.55	0.44
PEG-20	0.56	0.56

Note. The % nitrogen found is compared to the % nitrogen predicted from the structures of activated PEG shown in Fig. 1.

concentrations of activated PEG-5 on SOD derivitization is shown in Fig. 2. The nature of the PEG-protein adduct varies with the reaction conditions. Altered electrophoretic behavior probably reflects both decreased positive charge and increased size of the modified protein. The greater mobility electrophoretic bands in gels B, C, and D of Fig. 2 should represent unmodified and less highly modified protein.

Activity and clearance of PEG-modified SOD. The catalytic activity of SOD after modification with PEG-4 and PEG-5 was de-

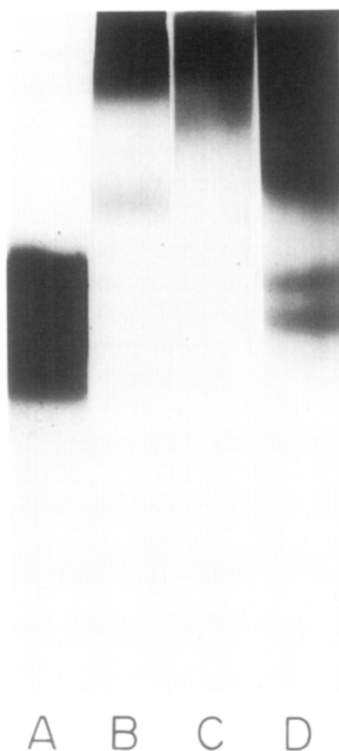


FIG. 2. Polyacrylamide gel electrophoresis under native conditions (24) of SOD and its PEG-5 derivative. Twenty micrograms of protein was applied to each gel lane. Gel A—unmodified SOD; gel B—SOD incubated with 90 mM activated PEG-5 for 48 h; gel C—SOD incubated with 90 mM activated PEG-5 for 48 h at which time the concentration of PEG-5 was doubled and the incubation continued for another 48 h; gel D—SOD incubated with 180 mM activated PEG-5 for 96 h. The direction of migration is from top to bottom with bottom being the anode.

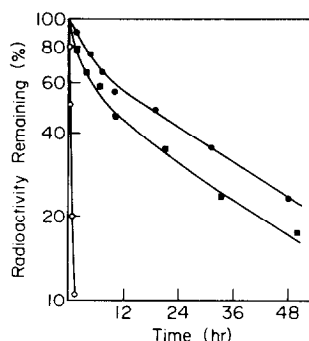


FIG. 3. Blood clearance in the mouse of SOD and its PEG derivatives. Graph shows the clearance of unmodified (O), PEG-5 (■), and the PEG-4-derivatized SOD (●).

termined. For both of these derivatives the enzyme retained greater than 95% of its activity. This compares to only 51% retention of enzymatic activity upon derivitization of SOD with cyanuric chloride activated PEG (8). The clearance of intravenously injected SOD and its PEG-4 and PEG-5 derivatives are shown in Fig. 3. Less than 15% of the native protein remained in the circulation 20 min after injection whereas approximately 20% of the PEG-5 derivative and 30% of the PEG-4 derivative remained in the circulation 40 h after injection. The apparent half-life of SOD was 3.5 min for the native protein, 9 h for the PEG-5 derivative, and 16.5 h for the PEG-4 derivative.

Activity of PEG-modified α_2M and α_2M -trypsin complex. The PEG-2, PEG-4, PEG-5, and PEG-20 derivatives of α_2M demonstrated trypsin binding activities of 80, 35, 38, and 63%, respectively, as compared to the unmodified protein. This decrease in trypsin binding activity may result from steric hindrance in the interaction of the PEG-modified α_2M with trypsin. There was, however, an increase in α_2M -trypsin amidolytic activity for each of the PEG derivatives of preformed α_2M -trypsin complex. For the PEG-2, PEG-4, PEG-5, and PEG-20 derivatives of α_2M -trypsin complex the amidolytic activity was 120, 120, 102, and 270%, respectively, of the



FIG. 4. Native polyacrylamide gel electrophoresis performed on 5% slabs according to Nelles *et al.* (23) of α_2 M-trypsin complex and its PEG derivatives. Five to seven micrograms of each protein sample was applied. Gel A—unmodified α_2 M—trypsin complex; gel B—PEG-2 derivative; gel C—PEG-4 derivative; gel D—PEG-20 derivative. The direction of migration is from top to bottom with the bottom being the anode.

control value. In control experiments, unreacted PEG had no effect on the activity of α_2 M—trypsin when added simultaneously with the substrate BAPNA. Trypsin amidolytic activity is increased after PEG derivatization using the cyanuric chloride procedure (35). The present results may represent a similar phenomenon.

Electrophoresis of PEG-modified α_2 M-trypsin complex. Figure 4 shows a comparison between the electrophoretic mobilities in a native electrophoresis system of unmodified and PEG-modified α_2 M—trypsin complex. The PEG derivatives of α_2 M—trypsin complex distributed in a broader band than unmodified α_2 M—trypsin suggesting some heterogeneity in the population of derivatives. The de-

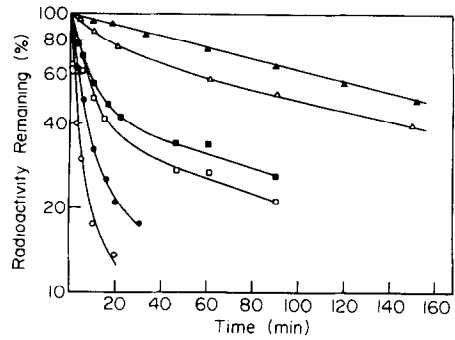


FIG. 5. Clearance of α_2 M—trypsin complex, and its PEG derivatives. The graph shows the clearance of unmodified α_2 M—trypsin complex (O), PEG-2 (●), PEG-4 (■), PEG-5 (□), and PEG-20-derivatized α_2 M-T (Δ). Native α_2 M (▲) is shown for comparison.

creased mobilities of the derivatives most likely reflect decreased positive charge and increased size as was the case for SOD.

Clearance of PEG-modified α_2 M-trypsin complex. Figure 5 shows the clearance of α_2 M—trypsin complex before and after reaction with activated PEG. The clearance of unmodified α_2 M—trypsin complex was rapid in agreement with previous studies (19,34). The PEG derivatives of α_2 M—trypsin complex cleared at a decreased rate. Reactions with higher molecular weight PEGs resulted in a more pronounced inhibition of clearance. The apparent half-life of the PEG-20 derivative approached that of the unreacted inhibitor.

Clearance of PEG-modified lactoferrin. Figure 6 shows the clearance of native and PEG-

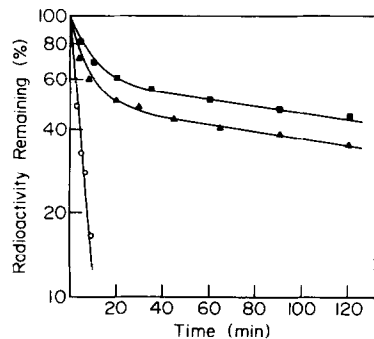


FIG. 6. Clearance of native and PEG modified lactoferrin. The graph shows clearances of unmodified (O), PEG-2 (▲), and PEG-4 derivatives of lactoferrin (■).

modified lactoferrin. The half-life increased from less than 3 min for unmodified lactoferrin to 15 min for the PEG-2-modified protein and to 60 min for the PEG-4 derivative. Unlabeled native lactoferrin significantly competed for clearance with the PEG-2 derivative of radiolabeled lactoferrin. When a 3000-fold molar excess of unlabeled native lactoferrin was injected with the radiolabeled derivative, the clearance of the derivative was prolonged fivefold. This result suggests that the derivatized protein clears on the same hepatocyte receptor that clears native lactoferrin (17). Lactoferrin precipitated when incubated with 40 mM PEG-5 or greater than 160 mM PEG-2 or PEG-4. Electrophoresis confirmed that the precipitates contained lactoferrin. Precipitated lactoferrin when redissolved cleared at a rate typical of the unmodified protein suggesting that no derivatization occurred. PEG is capable of precipitating many proteins without denaturation (36) and it appears likely that this phenomenon was observed under these reaction conditions.

DISCUSSION

Proteins are increasingly important in clinical medicine as drugs, but many of these proteins must be obtained from nonhuman sources since insufficient amounts can be purified from available human tissue or, for example, as in the case of streptokinase, the protein is not synthesized by eukaryotic organisms (see Ref. (34) for a discussion of the uses of streptokinase). This raises the problem of protein antigenicity. A second problem encountered when proteins are used as drugs is the short half-lives of many of these preparations. As an example, streptokinase has a half-life of only about 15 min (34).

Two interesting characteristics of some PEG-protein adducts, as compared to the unmodified proteins, are an increase in blood circulation time and a decrease in antigenicity and immunogenicity (1,6). Unfortunately, the coupling of PEG to proteins often results in

substantial loss of activity. The coupling of cyanuric chloride activated PEG to SOD results in an approximate loss of 50% of the activity. Modification of L-glutaminase-L-asparaginase results in loss of almost 90% of its activity (6,10).

This report describes a new technique for linking PEG polymers to proteins by using an easily prepared carbonyldiimidazole-activated PEG. The formation and isolation of the activated PEG is a simple three-step procedure consisting of incubating carbonyldiimidazole with PEG in a nonaqueous solvent, dialyzing the product against water, and lyophilizing the preparation. The resultant "activated PEG" is an imidazole carbamate derivative of the hydroxyl group of the polymer as illustrated in Fig. 1 for a monofunctional PEG. Support for this proposed structure was obtained by elemental analysis.

The proteins derivatized with the activated polyethylene glycols were chosen to represent three different mechanisms of plasma clearance; namely, passive excretion (SOD), recognition of unique amino acid recognition sites by a receptor (α_2 M-trypsin), and carbohydrate recognition by receptors (lactoferrin) (15,17,19). Two of these proteins have the additional advantage of possessing an enzyme activity (SOD) and a protease binding function (α_2 M), which can be evaluated after PEG modification. The antigenicity or immunogenicity of PEG adducts prepared by use of carbonyldiimidazole-activated PEG was not evaluated. These properties are not predictable a priori and must be evaluated for each PEG-protein adduct prepared by any of the reported coupling procedures.

When SOD was incubated with a 3600-fold molar excess of activated PEG-4 or PEG-5, less than 10% of the free amino groups remained. This compares favorably with cyanuric chloride-activated PEG-5 derivatization of SOD (8). The PEG-4 adduct of SOD made with carbonyldiimidazole-activated PEG had greater than 95% of the activity of native SOD and a blood circulation half-life

of 16 h. The PEG adduct of SOD made with cyanuric chloride-activated PEG had a similar half-life, but as described above, retained only 51% catalytic activity (8).

There was also a striking change in the blood circulation half-life of lactoferrin after derivatization with carbonyldiimidazole activated PEG. Native lactoferrin competed for the clearance of a radiolabeled PEG-lactoferrin complex consistent with the clearance of the modified derivative by the same mechanism which clears the native protein, albeit at a slower rate.

The ability of PEG-modified α_2 M to bind trypsin was decreased significantly, presumably because of steric hindrance in the interaction of the PEG modified α_2 M with trypsin. PEG modification of preformed α_2 M-trypsin, however, yielded derivatives which did not demonstrate loss of amidolytic activity. Clearance studies of α_2 M-trypsin demonstrated that a relatively large polymer (PEG-20) was necessary to cause an increase in the plasma half-life in contrast with the PEG derivatives of SOD and lactoferrin. The plasma clearance of PEG-20- α_2 M-trypsin actually approached that of the native molecule.

The plasma clearances of the PEG adducts of SOD, lactoferrin, and α_2 M-trypsin were generally biphasic while the clearances of the native ligands were essentially first order. This difference probably reflects some heterogeneity in the extent of protein modification. The comparisons, therefore, of the half-lives of modified and unmodified proteins is somewhat misleading since it tends to underestimate the actual increase in the circulating half-lives of the modified proteins. This was particularly evident for the PEG derivatives of lactoferrin where the apparent half-life increased from about 3 min for the reactive molecule to 15 min (PEG-2) and 60 min (PEG-4), respectively. In each case, 50 to 75% of the modified protein population actually demonstrated a plasma clearance of several hours.

These studies document the utility of carbonyldiimidazole-activated PEG as a cou-

pling reagent for proteins. This procedure offers a simplified approach for preparing such derivatives. The method may be particularly useful where other such procedures result in protein inactivation.

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