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The preparation and properties of novel reversible polymer-protein conjugates

2-ω-Methoxypolyethylene (5000) glycoxymethylene-3-methylmaleyl conjugates of plasminogen activators

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The preparation of a reagent capable of reversibly attaching polyethylene glycol to proteins and the use of this material in modifying the plasminogen activators urokinase- and tissue-type plasminogen activator are described. The characterisation and the reversible nature of these protein-polymer conjugates are discussed, and some of the in vitro properties of these modified enzymes are explored.

Urokinase; Plasminogen activator; Chemical modification

1. INTRODUCTION

Conjugation to the periphery of proteins with polyethylene glycol and other water-soluble proteins has proved to be a valuable way of inhibiting interactions with other macromolecules, thereby conferring on the protein advantages such as greater in vivo half-life and reduced susceptibility to proteases. Beneficial effects with respect to immunogenicity have also been exploited [1–6]. Where such conjugation has been applied to enzymes, it has been more successful with those that act on low molecular mass compounds [1,3], as presumably steric hindrance exercises an adverse influence on interaction with larger substrates. For example, in an attempt to moderate the immunogenicity of the streptokinase moiety, polyethylene glycol conjugates of streptokinase [7] and acylated plasminogen-streptokinase complex [8], two plasminogen activators of increasing importance in thrombolytic therapy, have been prepared. Whilst the desired effect of decreased immunogenicity was obtained, decreased activity was also observed.

The plasminogen activators of human origin, namely tissue-type plasminogen activator (tPA) and urokinase (uPA), whilst non-immunogenic in man, do suffer from a very rapid rate of elimination from the bloodstream [9,10]. A report of an irreversibly linked PEG-uPA conjugate [11] showed that the material resided for longer in vivo than the parent activator but suggested that a loss of activity had occurred. To circumvent the irreversible losses of activity associated with these approaches we have devised a protein-polymer linkage which is capable of breaking down in vivo. This strategy provides an inactive, relatively slowly cleared enzyme which is able to release active

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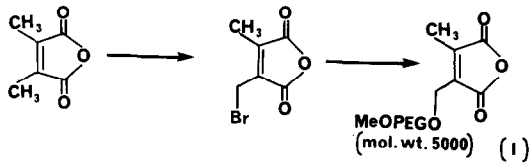

Scheme 1. Synthetic scheme for preparation of PEG-maleic anhydride reagent.

material at a reasonable rate thus obviating the clinical need to use long infusions, as, for example, is currently the therapeutic practice with tPA [12].

The reaction of amino groups with substituted maleic anhydrides [13,14] has been frequently used, especially for sequencing purposes [15]. Hydrolysis of the maleyl-amino peptide bond is facilitated by the neighbouring free carboxyl function [13]. After considering the deacylation kinetics of this system [16] we decided to prepare reagent (**I**) which is based on dimethylmaleic anhydride. An outline of the synthesis employed is given in scheme 1. This paper explores the use of this reagent to prepare reversible conjugates and reports on some of the properties of such conjugates with the plasminogen activators uPA and tPA.

2. MATERIALS AND METHODS

2.1. *Materials*

Methoxypolyethylene glycol, 5 kDa (Carbowax), was purchased from Union Carbide; other chemicals were purchased from Aldrich and used without further purification. Benzamidine agarose was supplied by Pierce. High molecular mass urokinase (uPA) was purchased from Serono (Freiburg, FRG). ^{125}I-uPA was prepared using the iodogen method, essentially as described by Fraker and Speck [17]. tPA, derived from cultured Bowes melanoma cells and purified using affinity chromatography on zinc chelate Sepharose and lysine Sepharose [18], was kindly supplied by Mr I. Dodd. Tripeptide chromogenic substrates S-2444 (uPA), S-2251 (plasmin) and S-2288 (tPA) were obtained from KabiVitrum, Sweden.

2.2. *Preparation of 2-[ω-methoxypolyethylene (5000) glycoxymethylene]-3-methyl maleic anhydride (I)*

(i) 2,3-Dimethylmaleic anhydride was treated with two equivalents of *N*-bromosuccinimide and a trace of benzoyl peroxide in boiling carbon tetrachloride for 22 h. After filtration, evaporation and distillation, a material was obtained (~40% yield) which was shown by ^{1}H NMR to be about 85% of the desired 2-bromomethyl-3-methylmaleic anhydride. Traces of dibromide and unbrominated material were also found. ^{1}H NMR ($CDCl_3$) 4.1 (2H,s,CH_2Br), and 2.2 (3H,s,CH_3).

(ii) To the sodium salt of methoxypolyethyleneglycol (5000) (from equivalent amounts of alcohol and sodium amide) in benzene, was added an equimolar amount of the material described above. After 22 h at reflux the solution was filtered and the product precipitated as a wax (85% yield) with petroleum ether. Difference infrared spectroscopy showed that this was mainly the title compound (ν_{max} 1770 cm) as opposed to an anhydride ring opened material. Titration of a hydrolysed sample of the wax with sodium hydroxide indicated that 1.08 mol anhydride per mol of PEG were present.

2.3. *Preparation of 2-[ω-methoxypolyethylene (5000) glycoxymethylene]-3-methyl-maleyl conjugates*

2.3.1. uPA

To a stirred solution of uPA (1.8 mg/ml) and ^{125}I-uPA (0.2 μCi) in 0.1 M sodium pyrophosphate/HCl, 0.1% (v/v) Tween 80, pH 8.0, buffer (buffer P) (0.5 ml) at 0°C was added **I** (100 mg) maintaining the pH at 8.0 by addition of 0.1 M NaOH. The material was purified by application to a column of benzamidine-Sepharose (9.5 mm × 11 cm bed weight) equilibrated with 0.1 M NaCl, 0.05 M NaH_2PO_4/Na_2HPO_4, 0.01% (v/v) Tween 80, pH 7.4 (PST buffer). After the excess hydrolysed reagent had eluted, the buffer was changed to 0.5 M arginine, 0.5 M NaCl, 20 mM Tris, pH 7.4 (ARG buffer), whereupon the modified uPA eluted as a single peak (fig.1). The conjugate was stored frozen at −40°C.

2.3.2. tPA

This reaction was performed in a similar way except that 0.1 M 4-guanidinobutyric acid, a non-nucleophilic analogue of arginine which binds to tPA, was included with buffer P to aid solubility. The material was also gel filtered into PST buffer using Sephadex G25M (2 × PD-10 columns, Phar-

macia) before application to benzamidine-Sepharose. The final material was desalted into 100 mM NH_4HCO_3, 0.2% D-mannitol using 4 PD-10 Sephadex G25M columns, and then lyophilised.

2.4. *Preparation of dimethylmaleyl-tPA*

To a solution of tPA (~0.35 mg/ml) in buffer P (1.0 ml) was added 2.5 M dimethylmaleic anhydride (6 × 50 μl) in dry dioxane at ~5 min intervals to give ~140000 equivalents of acylating agent. The pH was maintained at 8.0 by periodical addition of 0.5 M NaOH. After reaction for a further 15 min the amidolytic activity had fallen to zero [19]. The product was isolated by gel filtration of a PD-10 Sephadex G25M column (Pharmacia) equilibrated in PST buffer. To a portion 20% glycerol was added, and deacylation at 37°C monitored by assaying portions against substrate S-2288.

2.5. *Plasminogen activation assay*

Into a cuvette was placed 10 mM S-2251 in 0.1 M triethanolamine buffer, pH 7.0 (25 μl), 10 mg/ml lys-plasminogen (KabiVitrum, Sweden and treated with aprotinin-agarose) (10 μl) and test activator at an appropriate concentration (5 μl). These were added to the cuvette without allowing the solutions to mix. To a blank cuvette was added the above solutions with the exception of the activator. The assay was initiated by addition of 0.1 M triethanolamine buffer, pH 8.0 (1 ml), to both cuvettes and the A_{405} (test vs blank) monitored. The initial slope of a plot of A against time^2 was taken as the measure of the rate of plasminogen activation.

2.6. *Fibrin plate assays*

Fibrin plate assays were performed essentially as described by Granelli-Piperno and Reich [20].

2.7. *HPLC*

Analytical gel filtration HPLC was performed on a column (7.5 × 600 mm) of TSK G3000 SW equilibrated with 0.08 M Na_2HPO_4/NaH_2PO_4, 0.32 M NaCl buffer, pH 7.0, containing 20% ethanol [21] using a flow rate of 0.75 ml/min. Molecular mass calibration was achieved with standard proteins from Biorad (CA, USA).

3. RESULTS AND DISCUSSION

The half-life for the return of amidolytic activity of dimethylmaleyl tPA, was found to be 42 ± 2 min (SE, n = 4) by S-2288 assay. Consideration of the pharmacokinetics and pharmacodynamics of thrombolytic agents [22] suggested that this rate of regeneration was within a useful range and prompted the preparation of polyethylene glycol anhydride (**I**).

The reaction conditions chosen for the conjugations were based on those used for maleic anhydride type reactions [13], except that the concentrations of the PEG reagent were fairly low due to its high molecular mass.

Despite this, it was possible to prepare conjugates whose gel filtration molecular masses were substantially higher than that of the starting materials, being between 130 and 170 kDa. As each ethylene glycol moiety is reported to be triply hydrated [23,24], the actual molecular mass and degree of substitution of the conjugates may be somewhat less than these figures suggest.

Purification of both conjugates was achieved on

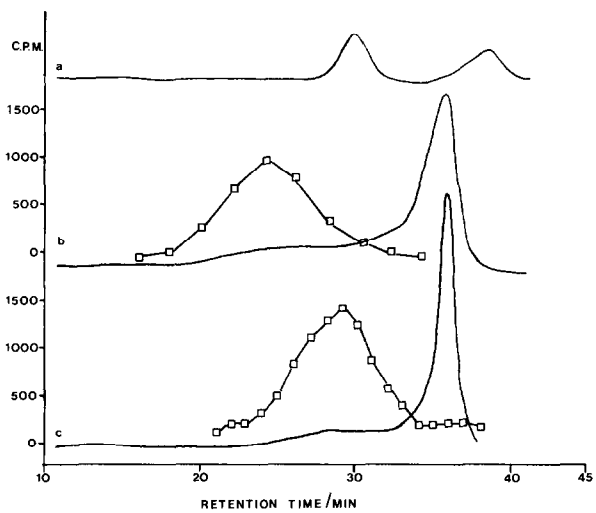

Fig.1. TSK G3000SW gel filtration HPLC profiles of (a) uPA, (b) the PEG-uPA conjugate, (c) the PEG-uPA conjugate after incubation at pH 7.4, 37°C for 72 h in 0.1 M NaH_2PO_4/Na_2HPO_4, 0.1 M NaCl, 0.01% (v/v) Tween 80, pH 7.4:glycerol, 4:1. The apparent molecular mass of the conjugate (170 kDa) has almost completely reverted to that of free uPA (53 kDa) by this treatment. (——) A profile; (□—□) ^{125}I profile.

an affinity column of 4-aminobenzamidine agarose [25] and gave good recoveries of conjugates well separated from excess reagents (not shown).

Loss of polymer from the PEG-uPA and PEG-tPA conjugates showing the reversible nature of these materials is shown in figs 1 and 2. The rate of polymer removal cannot be calculated accurately from these data.

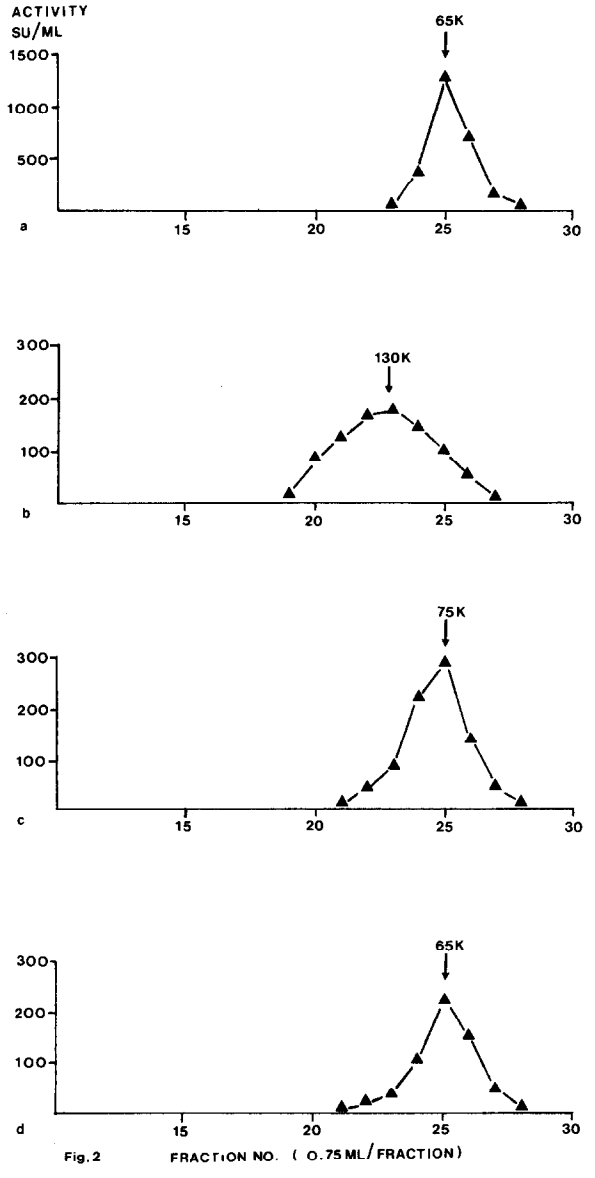

Fig.2. TSK G3000SW gel filtration HPLC amidolytic activity profiles of (a) tPA, the PEG-tPA conjugate after incubation at pH 7.4, 37°C for (b) 0, (c) 21 and (d) 44 h.

Fig.3 shows the rate of regeneration of functional enzyme in the plasminogen activation assay for the uPA conjugate. The initial activity of this conjugate was 44% that of the starting material. This process followed approximately first order kinetics with a half-life of 6.1 h. The fibrin plate assay of this conjugate, which takes ~16 h at 37°C, i.e. at least two half-lives for polymer removal, showed that the ratio of the specific fibrinolytic activity to the specific amidolytic activity (IU/SU ratio) of the conjugate was identical to the starting uPA, again implying that deacylation gives the native activator.

The tPA conjugate was 50–70% as active as unmodified activator in the plasminogen activation assay. Despite HPLC evidence of polymer removal (fig.2), no consistent time-dependent increase was noted possibly due to the instability of tPA under the reversing conditions. The fibrin plate activity (IU/SU ratio) was 77% that of the native tPA.

The pharmacokinetic properties of the uPA and tPA conjugates have been studied and results suggest that the PEG-uPA conjugate is cleared in the

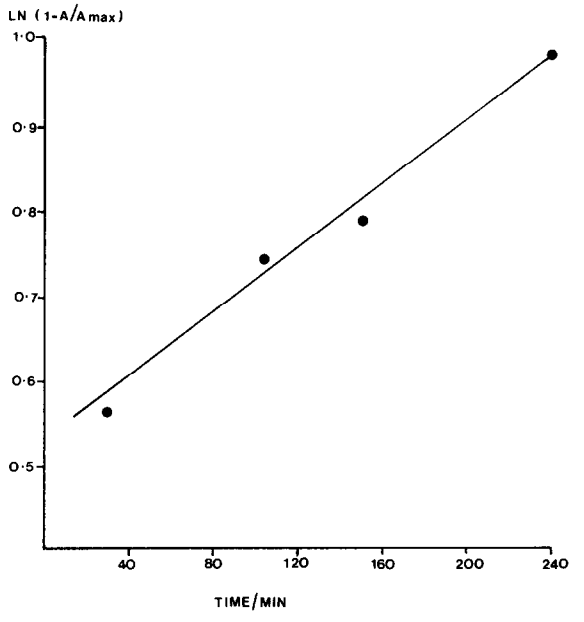

Fig.3. First-order plot of the regeneration of plasminogen activating activity of the PEG-uPA conjugate upon incubation at pH 7.4, 37°C.

guinea pig about 10-times more slowly than unmodified uPA [26] and the tPA conjugate about 5-times as slowly as the parent activator (unpublished).

In summary, we have demonstrated that reversible conjugation of proteins can be achieved with a PEG-containing maleic anhydride type reagent. As the number of proteins of therapeutic importance increases, this is potentially a generally applicable and non-toxic method of avoiding or delaying undesirable interactions with other macromolecules or cell surfaces, without rendering the protein permanently biologically inactive. For each application there will be a different optimum regeneration half-life, and it should be possible to alter this rate by changing the nature of the chemical groups attached to the double bond of the maleic anhydride. The scope of this approach remains to be fully delineated.

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