INVESTIGATION OF DRUG-PROTEIN INTERACTIONS AND THE DRUG-CARRIER CONCEPT BY THE USE OF BRANCHED POLYPEPTIDES AS MODEL SYSTEMS. SYNTHESIS AND CHARACTERISATION OF THE MODEL PEPTIDES*

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Synthesis of branched polypeptides with a new design of variations in surface topography is described. The side chains of the poly(Lys-(DL-Ala_m)) were elongated by single amino acids (L-Tyr, L-Glu, L-His, L-Leu, L-Pro, L-Phe) or short polymers (L-, D-Glu, L-Tyr, L-, D-, DL-Lys). Single amino acids were coupled, *via* the azide, active ester or N-carboxy anhydride method, oligomers were grafted by the polymerisation of N-carboxy anhydride derivatives. The resulting polypeptides were characterised by amino acid analysis, identification of the N-terminal residue of the chain ends, determination of the sedimentation coefficient and molecular weight estimation, based on sedimentation experiments and thin layer gel chromatography.

The augmenting effect of macromolecules on cytotoxic drug action has been established by a number of experiments[1-3]. The potentiation of antitumor activity by macromolecular carriers can be due to a preferential uptake of the combination in tumor cells *via* endocytosis[4]. Protective enveloping of the drug by the macromolecule might also be an important function of the carrier[5]. Combination with macromolecules can change the pharmacokinetics[2] and immunosuppressive activity of the drug. An important aspect is the possibility of a non-specific stimulation of the immune system by the carrier, as a synergistic effect. The role of macromolecular conformation in the binding process and biological properties is also an important problem to be investigated. These different aspects were selected to demonstrate the need for further research in order to establish the actual mechanism of action of macromolecular carriers.

For a considerable time we have been interested in the macromolecular carrier principle using protein-type carriers linked by covalent or non-covalent bonds to antitumor drugs[6-8]. In order to elucidate the factors determining favourable combinations the use of synthetic model compounds as carriers seemed to be a good

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approach. To simulate high molecular weight proteins a compact molecule of smaller dimensions would seem to be a better model than linear polyamino acids. Branched polypeptides (also called multichain polyamino acids) open up new possibilities. Synthetic branched polypeptides with poly(lysine) backbone have been previously used with success as protein models in immunological studies[9-11]. These structures can be modified easily in many ways (charge, size, stereochemistry, shape, immunogenicity *etc*), particularly by varying the outside determinants.

We are suggesting a new line of investigation in which they may be used with advantage. It is attempted to get a better understanding of the role of protein carriers in the potentiating of antitumor drugs. The model system seems to be suitable also to get further information on the molecular mechanism of protein-drug interaction in respect of the chemical nature of groups participating and forces prevailing. This problem is closely related to the transport of drugs linked non-covalently to protein carriers[12].

Model polypeptides used in our experiments were composed of two moieties: the inside area being a poly(L-lysine) backbone with short poly(DL-alanine) side chains grafted to the ε-amino groups, and the outside determinants, which consisted of single amino acids (Tyr,Glu,His,Leu,Pro,Phe) or their oligomers (2–6 residues). In the present paper synthesis and characterisation of some physicochemical properties of these substances are described.

EXPERIMENTAL

Materials*

All amino acids used for these studies were purchased from Reanal (Hungary), except D-lysine monohydrochloride (Fluka) and D-glutamic acid (Ajinomoto).

Intermediates

The following amino acid derivatives were synthesised using procedure described in the literature (amino acids are of L-configuration unless otherwise stated): N^{ε}-benzyloxycarbonyllysine[15], N^{α}-carboxy-N^{ε}-benzyloxycarbonyllysine anhydride[16], N^{α}-carboxy-N^{ε}-benzyloxycarbonyl-DL-lysine anhydride[17], N^{α}-carboxy-N^{ε}-benzyloxycarbonyl-D-lysine anhydride[18], N-carboxy-DL-alanine anhydride[19], N-benzyloxycarbonylhistidine hydrazide[20], N-benzyloxycarbonyltyrosine hydrazide[20], N-benzyloxycarbonylproline pentachlorophenyl ester[21], N-benzyloxycarbonylleucine pentachlorophenyl ester[21], N-carboxyphenylalanine anhydride[19] γ-benzyl N-carboxyglutamate anhydride[22], γ-benzyl N-carboxy-D-glutamate anhydride[22], N-carboxy-O-benzyloxycarbonyltyrosine anhydride[23].

* Abbreviations used in this paper follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature[13] in consistence with the recommended nomenclature of graft polymers[14].

Synthetic Procedures

Poly(L-lysine) was prepared from N^{α}-carboxy-N^{ε}-benzyloxycarbonyllysine anhydride. Conditions for polymerisation were chosen to obtain a degree of polymerisation of approx. 100—300 (ref.[22]). Removal of the protecting groups was achieved by HBr in acetic acid[24] (35%). Polylysine hydrobromide was precipitated with ether, dissolved in water and purified by exhaustive dialysis against water, using Visking casing and finally freeze dried. The average degree of polymerisation of the sample was DP = 200. $\overline{M}_w$: 41·600 ± 5% was calculated from data obtained by sedimentation equilibrium measurements.

Poly(Lys-(DL-Ala_m))

Poly(DL-alanine) side chains were grafted to the ε-amino groups of poly(L-lysine) according to[25]. Two different input amino-acid ratios were chosen (Lys : Ala = 1 : 3·65 and 1 : 10; Table I).

Poly(Lys-(X_i-DL-Ala_m)), $i < 1$, $m \sim 3$

Procedure A1: Benzyloxycarbonylhistidine hydrazide was previously coupled *via* the azide method to a branched polypeptide, poly(Lys-(polyAsp(OBzl))[24]. In our experiments the non-aqueous method of Honzl and Rudinger, using dimethylformamide as solvent[26], has been favoured because of solubility problems. The removal of benzyloxycarbonyl groups and purification of the end product has been achieved analogously to the methods used in the preparation of poly (lysine hydrobromide). The same procedure was applied for all polymers.

The amino acid analysis presented in Table I indicate, that relatively few side chain ends reacted under these experimental conditions.

Procedure A2: Adopting of the procedure according to Gillessen and coworkers[27] was more efficient (see amino acid analytical data in Table I). The protected amino acid hydrazide (10 mmol) in 90 ml dimethylformamide containing 20 ml 2M-HCl in tetrahydrofurane was cooled to —20°C and under vigorous stirring 2 ml isopentyl nitrite added. Stirring was continued for 30 min, the temperature adjusted to —30°C and 6·95 ml (50 mmol) triethylamine added, followed by the addition of the precooled 6% dimethylformamide solution of the poly(Lys-(DL-Ala_m)). (Input ratio: Lys-(DL-Ala_m): Z—X—N_2H_3 = 1 : 1·2 or 1 : 1·5.) Stirring was continued at 0°C for 2 h and the mixture kept for an additional 12 h at room temperature. By dilution using 10 volumes of water a precipitate was formed, filtered, washed with water and dried.

Procedure B: The method previously used for the modification of poly(L-lysine) by active esters[28,29] was adopted. A stirred 2% solution of poly(Lys-(DL-Ala_m)) in dimethylformamide was treated at room temperature with a 10% solution in dimethylformamide of the benzyloxycarbonylamino acid pentachlorophenyl ester (input molar ratio: Lys-(DL-Ala_m): Z-X-OPcp = = 1 : 1·5). Stirring was continued for 2 h; the mixture was kept at room temperature for 28 h. Dimethylformamide was evaporated *in vacuo* and the residue triturated by several portions of light petroleum, followed by trituration with ether. The solid material was dissolved in water, exhaustively dialysed against water (Visking casing) at 4°C, filtered and the clear solution freeze-dried.

Procedure C1: A 10% dioxane solution of N-carboxyphenylalanine anhydride was added in portions to a stirred 1% solution of poly(Lys-(DL Ala_m)) in pH 7 0·05M phosphate buffer at room temperature. (Input molar ratio: Lys-(DL)-Ala_m): anhydride = 1 : 1, buffer – dioxane ratio: 10 : 1.) Stirring was continued for 30 min and the reaction proceeded at room temperature overnight. The mixture was diluted with 4 volumes of water and dialysed for 86 h against several

changes of water at 4°C. The continuous removal of dioxane enabled the formation of a precipitate, which was finally filtered and dried.

Poly(Lys-(X_i-DL-Ala_m)), $i > 1$, $m \sim 3$

Procedure C2: Grafting of polymer chains to poly(Lys-(DL-Ala_m)) was performed as described previously[8] by adopting the method of Sela and coworkers[30].

The procedure was identical with C1, except that the N-carboxy-anhydride derivatives were applied in a 5% dioxane solution and the input ratio Lys-(DL-Ala_m): anhydride was higher as indicated in Table I.

The isolation was also somewhat different from procedure C1, since by the addition of 2 volumes of dioxane a precipitate could be formed, which was filtered, washed with dioxane and dried.

Removal of benzyl and benzyloxycarbonyl groups were performed as described[24].

Methods

Amino acid analysis were carried out on a Chinoin Model OE 975 analyser. The samples were subjected to hydrolysis with 6M hydrochloric acid in sealed tubes at 105°C for 24 h. Complete removal of benzyloxycarbonyl and benzyl blocking groups was controlled by the ultraviolet and infrared absorption spectra[24], using Specord UV VIS and Specord IR recording spectrophotometers. The identification of the terminal amino acids of side chains was realised by the adaptation of the method of Hartley[31]. A similar method has been previously used for the analysis of branched polypeptides applying 2,4-dinitrofluorobenzene instead of 1-dimethylamino--5-naphthalenesulfonyl chloride[25]. Derivatives containing N-terminal histidine residues have been localised with the Pauly reagent[32].

Sedimentation analysis was carried out in a MOM 3170 ultracentrifuge at 25°C with the schlieren optical system. The samples were sedimented in 0·9% NaCl solution at 50000 rpm, using a 3° single sector cell. Sedimentation coefficients were measured at 1 mg cm^{-3} concentration.

Thin layer gel chromatography was performed with a Pharmacia TLG apparatus, using Sephadex G 150 Superfine gel in 0·6 mm layers, equilibrated in 0·5M-NaCl for 24 h. 5 µl of a solution containing 10 mg per cm^3 polymer was applied by Finnpipette II. The time of development lasted 2 h at a 20° angle. Detection was performed by the replica technique, *i.e.* sample substances were transferred from the gel layer to a Whatmann 3MM sheet and the spots made visible by the aid of chlorine–tolidine or potassium iodide–starch reagents.

Molecular Weight Calculations

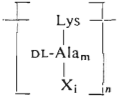
The average molecular weights were calculated in two ways.

The first approach was based on the determination of the molecular weight of poly(L-lysine) by sedimentation equilibrium. The partial specific volume of the lysine residue was taken as 0·72 (ref.[33]). Molecular weights of the branched polypeptides were calculated from the average degree of polymerization of the poly(L-lysine) backbone, n, multiplied by E, the equivalent weight of side chain per one lysine residue. The value of E was obtained from the quantitative amino acid analysis of residues in the side-chains ($i + m$) (ref.[24]).

The second approach was based on the thin layer gel chromatography experiments, using ribonuclease (13500; Reanal) bovine serum albumin (66500; Sigma) γ-globulin (160 000; Calbiochem) as standards.

RESULTS AND DISCUSSION

A number of branched polypeptides were synthesised with a poly(L-lysine) backbone and short poly(DL-alanine) side chains. The ends of the side chains, as outside determinants, were built up from a single residue or a short polymer of one particular amino acid, corresponding to the general formula



$n = 200$

$m \sim 3{\cdot}5$ (or $8{\cdot}6$)

Group I: $i < 1$

Group II: $i > 1$ $(\sim 2-3)$

Single amino acids were coupled to the chain ends by the azide, active ester or N-carboxy anhydride method. These techniques ensured that 70–80% of the N-terminal residues were identical. Polymers were grafted to the side chains by the polymerisation of N-carboxy anhydrides.

The products were characterised and their properties are in Table I. The sedimentation pattern was consistent with the presence of one component, giving a symmetrical schlieren peak. This finding is in agreement with the results obtained by thin layer gel chromatography. We would like to call attention to the differences in values of the sedimentation coefficients and their increase in cases the side chains consisted of polymers. This problem has been previously discussed by Yaron and Berger[24]. It has been reported, that for a molecular weight of 200000 a branched polymer (with relatively long polymer side chains) had a sedimentation coefficient larger by the factor of 3 than the value published for poly(N^{ε}-benzyloxycarbonyl-L-lysine) of the same molecular weight. Diffusion coefficients were found larger by the factor of 2 and intrinsic viscosity smaller by the factor of 8. These observations seemed to justify the general conclusion that branched polypeptides have a relatively compact shape, resembling the globular proteins. In our experiments the values of sedimentation coefficient were found always much higher in the second group of polymers ($i > 1$, polymer side chains ends) than in the first group ($i < 1$, single amino acid side chains ends). Comparing the molecular weights obtained by the TLG method and the values calculated on the basis of sedimentation measurements a good correlation was found only when the value of the sedimentation constant was high, indicating that only the more compact structures are suitable for the molecular weight estimation by TLG.

The design of the derivatives presented in this paper was based on the observation of Sela and coworkers[18], that the amino terminal residue of the side chain is immunodominant. The length of the side chains was planned in view of the observation that the size of the specific combining region of the antibodies is such as to accomodate a maximum of 3–4 alanine (or other amino acid) residues[18].

TABLE I

Preparation and Characterisation of Branched Polypeptides

Polypeptide	Preparation		Molar ratio of amino acids in the end product			$\overline{M}_w$ [a] (±5%)	$\overline{M}_w$ [b] (TLG)	$S_{20,w}$ (. 10^{13})
	procedure	polymer/monomer molar ratio	Lys	m	i			
poly(Lys-(DL-Ala_m))	C2	1 : 3·65	1	3·1	—	69 600	—	2·4
poly(Lys-(DL-Ala_m))	C2	1 : 4·1	1	3·45	—	73 900	—	2·86
poly(Lys-(DL-Ala_m))	C2	1 : 10	1	8·56	—	146 300	—	4·35
poly(Lys-(His_i-DL-Ala_m))[c]	A1	1 : 1	1	3·3	0·2	80 500	—	—
poly(Lys-His_i-DL-Ala_m))	A2	1 : 1·2	1	2·9	0·6	102 200	—	4·15
poly(Lys-(His_i-DL-Ala_m))	A2	1 : 1·5	1	3·4	0·8	112 200	—	4·48
poly(Lys-(Tyr_i-DL-Ala_m))[c]	A1	1 : 1	1	3·4	0·5	98 100	—	—
poly(Lys-(Tyr_i-DL-Ala_m))	A2	1 : 1·5	1	3·66	0·8	117 000	—	4·7
poly(Lys-(Pro_i-DL-Ala_m))[c]	B	1 : 1·5	1	3·1	0·5	100 100	—	3·4
poly(Lys-(Leu_i-DL-Ala_m))[c]	B	1 : 1·5	1	3·0	0·7	106 000	—	3·3
poly(Lys-(Phe_i-DL-Ala_m))	C1	1 : 1	1	3·4	0·66	94 600	—	5·1
poly(Lys-(Lys_i-DL-Ala_m))[c]	C2	1 : 3·5	1	3·1	3·4	227 000	208 000	4·71
poly(Lys-(D-Lys_i-DL-$Ala)_m$))[c]	C2	1 : 3·5	1	3·4	3·1	219 700	198 000	6·2
poly(Lys-(DL-Lys_i-DL-Ala_m))[c]	C2	1 : 3·6	1	3·1	3·1	215 400	213 400	5·96
poly(Lys-(Glu_i-DL-Ala_m))	C2	1 : 2·9	1	3·3	2·8	146 100	127 000	6·48
poly(Lys-(Glu_i-DL-Ala_m))	C2	1 : 6·7	1	3·4	6·3	236 400	—	—
poly(Lys-(D-Glu_i-DL-Ala_m))	C2	1 : 3·35	1	3·3	3·23	156 400	168 000	8·1
poly(Lys-(Tyr_i-DL-Ala_m))[c]	C2	1 : 2	1	3·2	1·9	147 400	128 000	—

[a] Calculated from the number average degree of polymerisation of poly-lysine and the side chain composition; [b] calculated from thin layer gel chromatography measurements; [c] isolated as hydrobromide.

The purpose of the synthesis of these polymers was to obtain a groups of compounds in which the side chain ends are covered in various degree by one particular amino acid. Interest was devoted also to the synthesis of analogues containing D- and DL-amino acids at side chains. It was hoped that these polypeptides might serve as suitable models for the study of the influence and the role of individual amino acids on the conformation of the polymers, – since very little is known about the conformation of branched polypeptides[34].

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