Preparation and Properties of Amphipathic Enzyme–Polymer Conjugates

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Amphipathic enzyme–polymer conjugates were prepared by reaction of hydrophilic enzymes with an anhydride polymer partially substituted with pendant hydrophobic groups. The products formed non-covalent aggregates of high molecular weight, dissociable by non-ionic detergents and urea and subject to additional aggregation at high ionic strength. Oil-in-water emulsions formed in the presence of such conjugates were shown to possess enzyme activity associated with the surface of the water-immiscible liquid. Fluorochrome labelling of conjugates showed that binding occurred at the surface of solvent droplets, and the conjugates were also found to aggregate liposomes.

The amphipathic properties of denatured (random-coil) proteins have been well known for many years and have important industrial implications (Phillips, 1977). It has recently become clear that many native proteins, particularly those associated with cellular membranes, also have amphipathic structures essential to their functional roles (Singer & Nicolson, 1972; Singer, 1974). At least three types of amphipathy have been distinguished in such proteins. In the first, a terminal section of polypeptide chain contains an abnormally high proportion of hydrophobic residues so that this domain can be embedded in the lipid bilayer with the hydrophilic portion of the protein exposed to the aqueous environment. Well-characterized examples of such intrinsic membrane proteins include cytochromes c_1 and b_5 (Trumpower & Katki, 1975; Spatz & Strittmatter, 1971, 1973) and glycophorin (Segrest *et al.*, 1973; Brulet & McConnell, 1976).

A second form of amphipathy arises through an asymmetric distribution of hydrophobic side chains across an α -helix, resulting in hydrophobic and hydrophilic faces rather than domains. Such a feature has been found in Amyloid A (Segrest *et al.*, 1976) and may be responsible for the lipid associating regions in lipoproteins (Segrest *et al.*, 1974). Thirdly, post-translational attachment of hydrophobic groups such as glycolipids may confer membrane-associating properties on a hydrophilic protein [as for example in the case of the *Bacillus licheniformis* penicillinase (Yamamoto & Lampen, 1975)].

I have already reported a technique for the controlled modification of hydrophilic proteins by covalent attachment to linear synthetic amphipathic polymers (Smith, 1976). Unlike hydrophobic modifications that involve indiscriminate linking of small lipophilic entities to the protein surface, the polymer conjugation technique produces material with protein and hydrophobic domains largely separate. In prin-

ciple it is possible to make modified proteins that retain functional characteristics such as enzyme activity, are analogous to the first type of naturally occurring amphipathy and have the surface-active characteristics normally associated with denaturation at interfaces. The present paper concerns the synthesis of two such conjugates, their physical properties and evidence for interactions with hydrophobic surfaces.

Materials and Methods

Brij 35, bovine trypsin (type III), *N*- α -benzoyl-DL-arginine *p*-nitroanilide, *p*-aminobenzamidine, fluorescein isothiocyanate/Celite, purified phosphatidylcholine and triethanolamine hydrochloride (Trien) were obtained from Sigma Chemical Co., Kingston on Thames, Surrey, U.K. Gantrez AN 119 was kindly donated by GAF (Great Britain), Wythenshaw, Manchester, U.K. *p*-Nitrophenyl *p*'-guanidinobenzoate hydrochloride was obtained from ICN Pharmaceuticals, Cleveland, OH, U.S.A. Cholesterol was obtained from British Drug Houses, Poole, Dorset, U.K. Compound S-2251 (D-valyl-leucyl-lysine *p*-nitroanilide dihydrochloride) was obtained from Kabivitrum, Ealing, London W5 2TH, U.K. Other solvents and reagents were commercial products of highest available purity used without further treatment.

p-Nitrophenyl phenylacetate was synthesized by the condensation of phenylacetic acid (0.05 mol) with *p*-nitrophenol (0.05 mol) by *NN'*-dicyclohexylcarbodiimide (0.05 mol) in dioxan (25 ml) for 16 h at 22°C. Dicyclohexylurea was filtered off and the filtrate evaporated and recrystallized from *n*-heptane/ethanol, giving white crystals, m.p. 65°C (yield: 72%).

n-Octadecylamine was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and recrystallized from ethanol before use. Partially purified *Escherichia coli* penicillin acylase was

supplied by Mr. T. Savage, Beecham Pharmaceuticals, Worthing, Sussex, U.K. [^3H]Acetic anhydride (0.5 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. *p*-Aminobenzamidine was coupled to Sepharose 4B by using the epoxide method of Sundberg & Porath (1974).

Cholesterol/phosphatidylcholine liposomes were prepared as follows. Cholesterol (20 mg) was dissolved in a chloroform solution (2 ml) containing purified egg-yolk phosphatidylcholine (Sigma type VII-E lecithin) (200 mg). The solution was evaporated *in vacuo* at a bath temperature of 47°C in a 30 ml pear flask. 0.1 M-Tris/HCl buffer, pH 7.4 (20 ml), pre-warmed to 47°C, was added and the mixture was dispersed by brief shaking. The preparation was sonicated under N_2 with a 9 mm probe sonicator (MSE) at 21 kHz in six 1 min bursts at a mean temperature of approx. 40°C. NaN_3 (0.05%) was added to the preparation, which was then stored under N_2 at 4°C.

The esterase activity of penicillin acylase was measured spectrophotometrically with 40 μM -*p*-nitrophenyl phenylacetate as substrate in 0.1 M-sodium veronal buffer, pH 8.3, at 25°C at 402 nm. The molar absorption coefficient for *p*-nitrophenolate under these conditions is 16600 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (Chase & Shaw, 1967). The K_m for the unmodified enzyme is 7.5 μM . Trypsin was assayed with as substrate 1.0 mM-*N*- α -benzoyl-DL-arginine *p*-nitroanilide in 0.1 M-Trien/HCl buffer, pH 8.0, at 25°C at 410 nm (Erlanger *et al.*, 1961) or compound S-2251 (see Fig. 2). Active-site titration was carried out as described by Chase & Shaw (1967). For insoluble hydrophobic preparations, enzyme/titrant mixtures were centrifuged after mixing and their absorbances read in the spectrophotometer against double blanks of centrifuged enzyme alone and titrant/buffer alone (prepared at the same time).

Polymer 18/25 (anhydride form) was prepared as follows. Gantrez AN 119 (10.0 g) was dissolved at *NN*-dimethylformamide (60 ml) at 80°C. A solution of *n*-octadecylamine (4.32 g) in dimethylformamide (40 ml at 80°C) was added, followed by pyridine (10 ml). After being stirred at ambient temperature for 18 h, the purple solution was added in two 60 ml lots to dry diethyl ether (2 $\times$ 500 ml at 4°C) and cautiously homogenized in a blender (MSE Ato-Mix). The precipitated gum was redissolved in dimethylformamide (40 ml) and precipitated again with dry diethyl ether (1.5 litres) at 4°C. The pink solid was filtered, washed with ether and dried *in vacuo*, yielding 4.83 g of amorphous product, which was stored desiccated at room temperature. P.m.r. ($[\text{D}_6]\text{dimethyl sulfoxide}$): broad singlet δ 1.20–1.40 p.p.m. (n-alkyl); envelopes, δ 2.5–2.9, 3.2–4.1 and 4.3–4.8 p.p.m. (backbone CH_2 , OCH_3 and CH); broad singlet, δ 9.0–10.0 p.p.m. (CO_2H exchanged with pyridinium); also pyridine peaks. The material (0.25 g) was dis-

solved in acetone (2.5 ml) and added to 0.1 M- Na_2CO_3 , pH 11.8 (25 ml). Hydrolysis of the polymer was monitored by titration with 0.1 M-NaOH and gave a mean value of 3.7 mequiv. of anhydride function/g.

Polymer 18/25 (hydrolysed form) was prepared as follows. Anhydride polymer 18/25 (0.5 g) was dissolved in dimethylformamide (2 ml) and added to 2 M-NaOH (17.5 ml) and stirred for 15 min at room temperature. The yellow flocculent precipitate was isolated by gentle centrifugation and dissolved in 0.5 M-glycylglycine, pH 8.5 (10 ml), and adjusted to pH 8.0. Then 5 ml of this solution was dialysed for 24 h against water (1 litre) at 4°C and freeze-dried, yielding 0.178 g of pale-yellow solid.

Trypsin-polymer 18/25 conjugates [conjugates T-18/25 (NaCl) and T-18/25 (H^+)] were prepared as follows. Bovine trypsin (0.5 g) was dissolved in 0.1 M-sodium veronal buffer, pH 8.3, and homogenized briefly with a solution of anhydride polymer 18/25 (0.5 g) in dimethylformamide (5 ml). The homogenization was carried out either in a pestle-type homogenizer (Tri-R Instruments, Rockville Centre, NY, U.S.A.) at 2000 rev./min at 0°C in four 15 s bursts or at low speed in a blender at the same temperature and for the same time. If a blender is used, care must be taken to avoid foam formation, since localization of the amphipathic polymer at the air/liquid interface gives intractable products, which are apparently cross-linked. The suspension was stirred overnight at 4°C and then adjusted to 4 M-NaCl by addition of solid salt. The mixture was centrifuged at 20000 *g* at 4°C for 1 h, and the resulting pellet was resuspended in 0.1 M-Trien/HCl/0.5% NaN_3 buffer, pH 8.0 (20 ml), and stored at 4°C [conjugate T-18/25 (NaCl)]. The supernatant was separated from a little surface scum and adjusted to pH 2.5 with 2 M-HCl. The precipitate was isolated by centrifugation as above, resuspended in 20 ml of the pH 8.0 buffer and stored at 4°C [conjugate T-18/25 (H^+)].

Penicillin acylase-polymer 18/25 conjugate (conjugate P-18/25) was prepared as follows. Anhydride polymer 18/25 (0.5 g) was dissolved in dimethylformamide (2.0 ml) and added to a solution of partially purified acylase (10 ml, adjusted to pH 7.7 in water; 3.21 μkat). The mixture was homogenized in Tri-R at 4000 rev./min with about 12 strokes at 22°C. The homogenate was stirred at 4°C for 3 h, then 0.1 M-Tris/HCl/0.1 M-NaCl buffer, pH 8.0 (10 ml), was added and stirring continued at 4°C for 72 h. The mixture was centrifuged at 15000 *g* at 4°C for 1 h, and the pellet was resuspended in the Tris/saline buffer (20 ml) and re-centrifuged. The final pellet was suspended in the same buffer (20 ml) and stored at 4°C. The specific activity was 42.3 nkat/ml.

$[\text{D}_6]\text{Acetylated conjugate P-18/25}$ (conjugate $[\text{D}_6]\text{Ac-P-18/25}$) was prepared as follows. Conjugate

P-18/25 (1.0ml) was dialysed against 0.1M-sodium acetate buffer, pH 7.0 (1 litre), for 16h at 4°C and then treated with [³H]acetic anhydride (5mCi; 10μmol) in dioxan (0.1 ml). The mixture was stirred at 4°C for 16h and then dialysed against two changes of 5 litres of acetate buffer over 48h at 4°C. This was followed by three changes of deionized water and resulted in a clear viscous solution with a specific radioactivity of 1.37×10^5 d.p.m./ml and a specific enzyme activity of 15.2nkat/ml.

[³H]Acetylated conjugate T-18/25 (H⁺) [conjugate [³H]Ac-T-18/25 (H⁺)] was prepared as follows. Conjugate T-18/25 (H⁺) (1.0ml) was dialysed as above and treated with [³H]acetic anhydride (7.5 mCi; 15μmol) in dioxan (0.15ml). After the dialysis sequence described above, the resulting solution had a specific radioactivity of 2.72×10^6 d.p.m./ml and a specific enzyme activity of 3.0nkat/ml.

Fluorescein isothiocyanate-conjugate T-18/25 (H⁺) conjugate [conjugate FITC-T-18/25 (H⁺)] was prepared as follows. Fluorescein isothiocyanate/Celite (1:9, w/w; 0.1g) was added to conjugate T-18/25 (H⁺) (1.5ml) at pH 8.0 and stirred for 2h at 4°C. 0.1M-Trien/HCl buffer, pH 8.0 (1.0ml), was added and the mixture centrifuged to 1000g for 5min at 22°C. The supernatant was applied to a Sephadex G-25 column (1.5cm × 7.0cm) equilibrated and eluted with the Trien buffer. Fractions eluted near the void volume were pooled and stored at 4°C (total volume 6.0ml).

Results and Discussion

Polymer synthesis and coupling conditions

My first attempts to construct hydrophobic synthetic 'tails' for native enzymes used soluble dextrans of defined molecular weight activated by CNBr. The activated dextrans were substituted with n-alkylamines and 1,6-diaminohexane, the latter acting as a source of free amino groups in the amphipathic polymer. Enzymes were coupled to the modified dextrans by using glutaraldehyde. The products, although surface-active in the sense that they adhered to dispersed hydrophobic liquids, could not be solubilized with detergents and formed aggregates at the interface of oil-in-water emulsions. The use of bifunctional reagents in the synthesis of conjugates based on maleic anhydride-methyl vinyl ether co-polymers also gave such cross-linked products. However, the latter co-polymers could be modified with hydrophobic n-alkylamines alone, leaving sufficient anhydride groups available in the anhydrous product to couple efficiently with enzymes. The basis molecular weight of the Gantrez AN 119 co-polymer is about 230000, and I have prepared derivatives with 10–40% substitution of the anhydride units by C₁₀–C₁₈ n-alkylamines. One of the most

useful polymers was Gantrez AN 119 substituted to a nominal extent of 25% by n-octadecylamine; this polymer is designated polymer 18/25. Modified polymers were isolated from dimethylformamide/pyridine reaction mixtures as amorphous pink solids by precipitation with diethyl ether, and they were found to be soluble in other polar solvents such as dimethyl sulphoxide, pyridine or acetone. The extent of substitution by hydrophobic groups was estimated by p.m.r. to be somewhat lower than the nominal substitution; for polymer 18/25 it is about 20%. The p.m.r. spectra of these anhydride polymers were consistent with random substitution of the anhydride units by the n-alkylamine groups, and it is unlikely that discrete sections of the polymer chain differ significantly in net hydrophobicity. These amphipathic polymers are therefore structurally analogous to monoglycerides polymerized via their hydrophilic head groups. Anhydrous polymer 18/25 contained approx. 3.7mequiv. of anhydride function/g, a value that suggests some hydrolysis of non-substituted anhydride units. Base hydrolysis of the substituted polymers gave materials varying from fully water-soluble (generally, less than 20% substitution by C₁₂ or lower n-alkylamine homologues) to highly aggregated water-insoluble polymers (more than 33% substitution by C₁₆ or higher n-alkylamine homologues). Polymer 18/25 was of intermediate hydrophobicity, and the behaviour of the conjugates derived from it was found to depend on degree of protein substitution.

Ether-precipitated anhydride polymers were found to be unsuitable for direct coupling to enzymes, since enzyme inactivation occurred during hydrolysis of the solid polymer in aqueous solution. This effect may have been due to denaturation of the protein at the hydrophobic anhydride polymer/ aqueous interface. Successful coupling required mixtures that were as nearly homogeneous as possible. This was achieved by dissolving the polymer in dimethylformamide and adding it to aqueous enzyme solution with efficient homogenization but avoiding foam formation. Soapy solutions resulting from this procedure were allowed to react until the pink colour of the anhydride polymer was completely discharged, either by extended incubation at slightly alkaline pH or by the use of hydrophilic nucleophiles such as glycine to substitute any remaining anhydride groups. It is important that such groups should be removed, since handling procedures such as freeze-drying may result in cross-linking. Table 1 shows yields in the coupling of bovine trypsin and *E. coli* penicillin acylase with polymer 18/25. Neither product was completely soluble in buffer solutions, and the trypsin conjugate was fractionated by precipitation from 4M-NaCl, which gave a hydrophobic component [conjugate T-18/25 (NaCl)]. Acidification of the supernatant to pH 2.5 gave a more hydrophilic

component soluble in neutral buffers [conjugate T-18/25 (H^+)]. Overall, the retention of enzyme activity and active-site integrity was reasonably high, and each conjugate was stable at 4°C in aqueous suspension for many months.

The coupling conditions were not optimized for retention of enzyme activity, and the yields given in Table 1 are lower than some of those found previously under rather different conditions (Smith, 1976). I have observed that increasing the hydrophobicity of a polymer by increasing the alkylamine chain length at a constant degree of substitution appears to result in a lower recovery of activity. This effect may be due to inactivation of the enzyme during the early stages of anhydride polymer hydrolysis, and may possibly be avoided by better homogenization techniques. Radiolabelling with [3H]acetic anhydride could be performed on both conjugates without significant loss of activity.

Physical properties of the conjugates

To show that the amphipathic conjugates were covalently linked and not merely the result of strong ionic and hydrophobic enzyme-polymer interactions, the water-soluble fraction conjugate T-18/25 (H^+) was subjected to affinity chromatography on immobilized *p*-aminobenzamidine (Sundberg & Porath, 1974). The conjugate was almost completely excluded from the gel (Fig. 1) and had an effective molecular weight comparable with the exclusion limit of the modified agarose. A control experiment showed that the affinity column was capable of separating mixtures of unmodified trypsin and hydrolysed polymer 18/25. The high molecular weight of the conjugate was confirmed by polyacrylamide-disc-gel electrophoresis, which showed that it barely entered 5% acrylamide gels. Such behaviour suggests that even water-soluble conjugates (nominal molecular weight 300 000–400 000) are either extensively cross-linked by protein or undergo significant non-covalent association in solution. The latter point was examined by agarose-

gel filtration in the presence of the non-ionic detergent Brij 35. Fig. 2 shows elution profiles of conjugate T-18/25 (H^+) on Sepharose 4B in the presence and absence of the detergent. A significant increase in the proportion of non-excluded material in the presence of the detergent suggests that the latter lowered the effective molecular weight of the amphipathic conjugate by disruption of intermolecular aggregates. This aggregation is probably mediated by the hydrophobic pendant groups and may involve micelle-like structures. The possibility that the more hydrophobic conjugates such as T-18/25 (NaCl) and P-18/25 are cross-linked macroreticular aggregates is unlikely in view of the marked decrease in the turbidity of conjugate suspensions caused by increasing concentrations of non-ionic detergents or urea (Fig. 3). Cross-linked aggregates may be formed if high concentrations of protein are present in coupling mixtures, but, if anhydride polymer 18/25 and protein are maintained at high dilution, the formation of insoluble material is not a problem.

The effect of ionic strength on equilibrium light-scattering from suspensions of conjugate P-18/25 is shown in Fig. 4. Between 0.5M- and 1.5M-NaCl, aggregation apparently increased, but was partly reversed at higher ionic strength. Aggregation would be encouraged by a decrease in intermolecular repulsion of the anionic polymer chains, and the gain in solvent entropy associated with hydrophobic intermolecular forces would predict a simple salting-

Fig. 1. Chromatography of conjugate T-18/25 (H^+) (approx. 36 nmol of trypsin) on a *p*-aminobenzamidine-Sepharose 4B column (1.5 cm × 30 cm) equilibrated and eluted with 0.1 M-Trien/HCl buffer, pH 7.0, at 4°C. The arrow indicates application of the same buffer containing benzamidine hydrochloride (2.0 mM). ○, A_{280} ; ●, amidolytic activity, measured with *N*- α -benzoyl-DL-arginine hydrochloride *p*-nitroanilide (as described in the Materials and Methods section) and shown as nmol of *p*-nitroaniline released/s.

Table 1. Recovery of enzyme activity on coupling anhydride polymer 18/25 to trypsin and to penicillin acylase

The activity of conjugate T-18/25 was determined by active-site titration, and that of conjugate P-18/25 by esterase assay. Experimental details are given in the Materials and Methods section.

Conjugate	Fraction	% of initial activity
T-18/25	NaCl precipitate	21.0
	Acid precipitate	8.4
	Acid supernatant	9.1
P-18/25	First pellet	26.7
	Supernatant	1.4
	Washed pellet	26.4
	Washings	0.4

Fig. 2. Gel filtration of conjugate T-18/25 (H^+) (21.3 nkat) on a Sepharose 4B column (1.2 cm $\times$ 15 cm) equilibrated and eluted at 4°C with 0.1 M-Trien/HCl buffer, pH 7.0, containing no detergent ($\bullet$), 2.5% (w/v) Brij 35 ($\circ$) or 5.0% (w/v) Brij 35 ($\blacktriangle$)

The fraction size was 0.6 ml. The elution volume of Blue Dextran (mol. wt. 2×10^6) was 6.8 ml. Amidolytic activity was measured with 0.25 mM-compound S-2251 in 0.1 M-Trien/HCl buffer, pH 8.0, at 25°C, the *p*-nitroaniline release being observed at 405 nm.

Fig. 3. Effect of non-ionic detergent ($\bullet$) and urea ($\square$) on light-scattering by conjugate P-18/25 (0.1 ml of stock suspension) in 0.1 M-Tris/HCl/0.1 M-NaCl buffer, pH 7.4 (Brij 35), and 0.1 M-Trien/HCl buffer, pH 8.0 (urea). Measurements were made in 3.0 ml cuvettes, of 1 cm path length, at 25°C.

Fig. 4. Effect of ionic strength on equilibrium light-scattering from conjugate P-18/25 (0.1 ml of stock suspension) in 0.1 M-Trien/HCl buffer, pH 8.0 (2.5 ml) at 25°C $\bullet$, A_{650} ; $\circ$, conjugate P-18/25 esterase activity; $\blacksquare$, unmodified enzyme esterase activity.

out effect. In contrast with results obtained with polyvalyl-ribonuclease (Nishikawa *et al.*, 1968), salting-out with NaCl was limited to a narrow concentration range. This effect may reflect intramolecular processes within the polysoap, for example a change from an extended structure capable of forming micelle-like aggregates to discrete globular arrangements. It may be noted that the decrease in coupled enzyme activity with increasing ionic strength parallels that of the free enzyme, suggesting that electrolyte-mediated changes in the amphipathic polymer do not themselves have profound effects on the catalytic centre.

Interaction of the conjugates with hydrophobic surfaces

The immobilization of amphipathic conjugates was investigated in four ways. First, the extraction of enzyme activity from an aqueous phase on to a conjugate-mediated emulsion of a hydrophobic liquid was compared with the interaction of unmodified enzyme with an emulsion formed from control polymer and the same hydrophobic phase. In these experiments the substrate for the droplet-immobilized enzyme was in either aqueous or hydrophobic phases. Secondly, radiolabelled conjugates were used to examine the conditions under which enzyme-bearing emulsions were stable to washing procedures. Thirdly, conjugates were found to aggregate sonicated phosphatidylcholine/cholesterol liposomes, resulting in precipitable phospholipid-conjugate complexes. Fourthly, fluorochrome-labelled conjugate was used to obtain fluorescence photomicrographs of conjugate-bearing solvent droplets. Table 2 shows the distribution of enzyme activity between aqueous and

hydrophobic phases when conjugate P-18/25 was subjected to a single homogenization with various liquids. Control emulsions were prepared by using the same activity of unmodified enzyme and hydrolysed polymer 18/25. It is clear that this single dispersion step effectively associates activity with the non-aqueous phase and that anionic droplets formed by emulsification with the control polymer do not absorb significant amounts of free enzyme. In a related experiment, the penicillin acylase substrate *p*-nitrophenyl phenylacetate was dissolved in *n*-decanol and an emulsion was formed with this solution and aqueous suspension of either conjugate P-18/25 or control polymer 18/25 with an equivalent activity of free enzyme. In both cases the droplet phase was washed and the release of *p*-nitrophenol monitored. Fig. 5 shows that the washed conjugate emulsion catalysed the hydrolysis of *p*-nitrophenyl phenylacetate whereas the control emulsion showed only slow hydrolysis.

When *n*-decanol droplets bearing conjugate [^3H]Ac-P-18/25 were washed with buffer alone little radioactivity was lost to the aqueous medium. Such transfer was produced by inclusion of sodium dodecyl sulphate in the buffer at concentrations well above the critical micelle concentration of this detergent. In this case sodium dodecyl sulphate probably maintains the emulsion by replacing the pendant hydrophobic chains on the conjugate that is itself transferred to sodium dodecyl sulphate micelles (Fig. 6).

Sonicated phosphatidylcholine/cholesterol mixtures were prepared under conditions similar to those used by Huang & Thompson (1974). Although the lipid preparation used was heterogeneous in the fatty acid component, the vesicles were formed at a temperature well above the melting range for phosphatidylcholine. The preparations were largely but not completely excluded from Sepharose 4B, suggesting that, on average, these liposomes were

larger than those obtained by more exhaustive sonication. These vesicle suspensions could not be sedimented by low-speed centrifugation. When such suspensions were mixed with conjugate [^3H]Ac-T-18/25 (H^+) the liposomes were partially aggregated and could be sedimented under conditions where neither liposomes nor conjugate alone were precipitated. Vesicles aggregated in this way could not be chromatographed on Sepharose 4B because their large size resulted in clogged columns. Fig. 7 shows

Fig. 5. Release of *p*-nitrophenol from emulsions of 10mM-*p*-nitrophenyl phenylacetate in decanol (1.0ml) formed by homogenization in 0.1M-sodium veronal buffer, pH8.3 (5.0ml), at 3000rev./min in Tri-R for 1.0min at 25°C with (a) unmodified penicillin acylase (○, 44.4nkat), (b) conjugate P-18/25 (■, 6.25nkat) or (c) conjugate P-18/25 (●, 28.1nkat)

Decanol droplets were washed by centrifugation from 0.1M-sodium veronal buffer, pH8.3 (5.0ml), at 2000g for 2.0min at 25°C and then resuspended in the same buffer (10.0ml), and *p*-nitrophenol was measured spectrophotometrically in the aqueous layer of samples taken from the stirred mixture at 25°C.

Table 2. Distribution of enzyme activity between organic droplet and aqueous phase after homogenization of amphipathic conjugate with water-immiscible liquids

Conjugate P-18/25 (10μg of polymer; approx. 2.1nkat of penicillin acylase activity) in 0.1M-sodium veronal buffer, pH8.3 (10ml), was homogenized at 10000rev./min in Tri-R at 22°C for 1min with solvent (1.0ml) and phases were separated by centrifugation at 2000g at 22°C for 1min. Droplet enzyme activity was measured by agitation of samples of droplet phase in the cuvette (chromogenic esterase assay).

Solvent	Control hydrolysed polymer 18/25 + unmodified acylase		Conjugate P-18/25	
	% of activity in aqueous phase	% of activity in droplets	% of activity in aqueous phase	% of activity in droplets
<i>n</i> -Heptane	88	4	29	21
<i>n</i> -Decane	89	1	20	34
<i>n</i> -Decanol	60	0	0	18
Toluene	76	0	17	18
Glycerol trioleate	90	0	0	27

Fig. 6. Distribution of radioactivity between aqueous (○) and *n*-pentadecane (●) droplet phases of an emulsion formed from conjugate [^3H]Ac-P-18/25 (15nkat), *n*-pentadecane (1.0ml) and 0.1M-Tris/HCl buffer, pH8.0 (5.0ml), by homogenization at 3000rev./min in Tri-R for 2.0min at 22°C

The droplets were washed with buffer (5.0ml) by centrifugation at 2000g for 5.0min at 22°C. The arrow indicates the application of wash buffer containing sodium dodecyl sulphate (20mM).

Fig. 7. Precipitation of conjugate [^3H]Ac-T-18/25 (H^+) (0.075nkat; 0.031 μCi) in the presence of cholesterol/phosphatidylcholine liposomes in 0.1M-Tris/HCl buffer, pH7.4 (1.0ml), at 25°C

The liposomes were prepared as described in the Materials and Methods section. After mixing, conjugate/liposomes were incubated at 25°C for 1h and centrifuged at 4000g for 15min at 22°C. ●, Pellet radioactivity; ○, supernatant turbidity.

Fig. 8. Fluorescence photomicrograph of the droplet phase of an emulsion formed from conjugate FITC-T-18/25 (H^+) (2.0ml), 0.1M-Tris/HCl buffer, pH8.0 (3.0ml), and *n*-pentadecane (0.25 ml), homogenized at 4000rev./min in Tri-R for 2.0min at 22°C
Total magnification $\times 1000$.

the percentage of precipitable radioactivity as a function of phospholipid concentration. The extent of complex-formation rose to a maximum corresponding to about 5000 molecules of phospholipid per trypsin active centre and then decreased slightly. If aggregation can be ascribed to cross-linking of the bilayer structures by the amphipathic polymer (and hydrolysed polymer alone causes a similar aggregation), then it is possible that in the presence of excess phospholipid the conjugate is predominantly associated with smaller liposome structures. Fig. 8 shows a fluorescence photomicrograph of conjugate FITC-T-18/25 (H⁺) homogenized with n-pentadecane. Surface fluorescence was not evenly distributed between droplets, but appeared to be fairly evenly disposed around the circumference of any one droplet. There was no evidence of macroreticular aggregates adhering to droplet surfaces, and it is unlikely that the conjugates penetrate or are entrapped inside the hydrophobic liquid bulk.

Conclusions

The basic assumptions about amphipathic enzyme-polymer conjugates, namely their non-cross-linked nature and surface-active properties, appear to be well founded. However, the scope and usefulness of the amphipathic conjugation technique have yet to be fully assessed. I have found that efficiency of conjugation, like most immobilization procedures, varies greatly from protein to protein, but a substantial number of enzymes from different sources may be conjugated without inactivation. It is unlikely that the types of polymer used in these experiments are optimal, and shorter more-hydrophobic stretches of polymer might be more efficient in achieving high loadings of enzyme activity on hydrophobic surfaces. Nevertheless these simple techniques have enabled me to construct a novel model enzyme reactor system, in which penicillin acylase activity immobilized on hydrophobic liquids is used to cleave aqueous benzylpenicillin continuously (Smith, 1977). In such a system, the enzyme-coated droplets are allowed to separate from aqueous product in a separate

'flotation' vessel, from which they are returned by pumping to a main stirred-tank reactor. A detailed analysis of the performance of this reactor has not been made, but preliminary work has shown that such systems can be made to operate continuously for significant periods of time, and that there is much scope for the development of this type of system, particularly where water-immiscible substrates are involved.

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