Microgels as Supports for α-Chymotrypsin

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α-Chymotrypsin is conjugated to carboxy-bearing microgels with a water-soluble carbodi-imide. The Michaelis–Menten parameters of the conjugated enzyme *versus* *N*-benzoyl-L-tyrosine ethyl ester are almost identical with those for the native enzyme except at low pH where coagulation of the microgel occurs. The enzyme covers *ca.* 9% of the 'wet' microgel surface. Control experiments with carboxymethyl-Sephadex as an insoluble analogue of the microgel indicate that the enzyme is deeply bound in the Sephadex particle consistent with altered Michaelis–Menten parameters of this conjugate.

The advantages in synthesis and analysis conferred on enzymes by immobilisation on insoluble supports have been exploited in recent years.[1] Enzymes immobilised on soluble polymeric supports should possess decreased resistance to diffusion enabling the immobilised species to act on polymeric or solid substrates. The soluble polymer matrix could also modify the solubility properties of the enzyme, for example solubilising it in organic media. Naturally occurring soluble polymers such as dextran have been tested as supports;[2] enzymes immobilised on linear synthetic polymers have also been studied.[3]

Recent work has shown that enzymes can be attached covalently to non-cross-linked synthetic latex particles[4] and to microgels.[5] These particles are prepared by emulsion polymerisation; the presence of a cross-linking monomer unit in the monomer feed ensures that the polymer particle has a structure relatively independent of solvent and such a particle is defined as a microgel.[6,7]

Previous work from this laboratory has demonstrated an enhanced reactivity of reagent groups attached to microgels compared with that of the corresponding small-molecule reagents.[7] Enhanced reactivity is possibly due to the group reacting within the microgel matrix away from the regular solvent. Covalent attachment of enzymes to microgels should only be possible on the surface of the particle. The attached enzyme should be accessible to solvent and its reactivity should not differ much from that of the native enzyme provided the polymer matrix does not absorb substrate or provide large electrical effects.

In the present study, chymotrypsin is covalently linked to water-soluble microgel particles containing carboxy groups. Michaelis–Menten parameters (K_m and k_{cat}) can be readily determined for the conjugated enzyme; the total amount of active enzyme is easily determined by titration with a substrate[8] owing to the transparent nature of the microgel sol. There have been no reports of quantitative physicochemical studies of enzymes attached to soluble polymers and this work is aimed at understanding the structure of the conjugated enzyme as revealed by any changes in catalytic properties.

Experimental

Materials.—The monomers acrylic acid, methacrylic acid, methyl methacrylate, 2-hydroxyethyl methacrylate, and ethylene glycol dimethacrylate were obtained from Aldrich and redistilled under vacuum to remove inhibitors. 4-Nitrophenyl acetate was prepared according to the method of Chattaway.[9] *N*-Benzoyl-L-tyrosine ethyl ester (BTEE), α-chymotrypsin (Type II), and 1-ethyl-3-(3-*NN*-dimethylaminopropyl)carbodi-imide HCl (EDC) were obtained from Sigma and used without further purification. Water used throughout the investigation was doubly distilled from glass.

Polymers.—The latex particles were prepared by a standard method.[7] The monomer feed (5 ml) was added to water (100 ml) with sodium dodecyl sulphate (100 mg) in a glass soda-bottle which was equilibrated to 70 °C in a Trigol bath on a heater–stirrer plate. The mixture was purged with nitrogen, emulsion polymerisation initiated with ammonium persulphate (50 mg), and the bottle sealed with its screw-cap. Polymerisation was stopped with quinol (50 mg) after the characteristic 'blue' opalescence had appeared but prior to formation of solid particles. The solution was centrifuged to remove any coagulated matter and cleaned from residual monomer by passage through a Sephadex G25 column; it was purged of residual surfactant by stirring with strong base macroreticular anion exchange resin (Amberlyst A 26) in its hydroxide form. An alternative purification procedure involved precipitation of the polymer with dilute acid, centrifugation, and solution by addition of dilute NaOH; the procedure was repeated several times and was followed by stirring the polymer with anion exchanger in its hydroxide form. In all cases the product polymer was tested for purity by passage through an analytical Sephadex G25 column.

Polymer feed composition in mol percentage was: methyl methacrylate, 45; 2-hydroxyethyl methacrylate, 30; ethylene glycol dimethacrylate, 10; methacrylic or acrylic acid, 15%. Yields were of the order of 100% as judged from recovered solids and the purified polymers essentially monodisperse as determined by electron microscopy and light scattering. Two polymers were employed in this work, one containing acrylic acid (polymer-1) and the other containing methacrylic acid (polymer-2). All the kinetic work was carried out using the *same* batch of each polymer.

Kinetic Methods.—A portion of a stock solution of 4-nitrophenyl acetate in acetonitrile (25λ, 10mM) was diluted to 2.5 ml in buffer at pH 7.8 in a silica cell in the cell compartment of a Perkin-Elmer Lambda 5 u.v.–visible spectrophotometer. When the temperature had equilibrated a portion of enzyme or enzyme–polymer conjugate was added on the tip of a glass rod and the recorder activated. The initial burst of absorbance at 400 nm was obtained by extrapolation and, together with the extinction coefficient for 4-nitrophenol at the wavelength and pH of the measurement, gave the molarity of enzyme active sites. In the case of the enzyme immobilised on CM-Sephadex, stirring was effected in the cell by an apparatus previously described.[10]

Kinetic studies with BTEE as substrate were carried out in a

similar fashion except that a pH range was employed and the wavelength was 254 nm up to pH 9.1 and then 294 nm. The extinction coefficient of the absorption change was measured for each run by simply allowing the substrate to hydrolyse completely; it varied from 1 060 at low pH to 541 at pH 9.1 for 254 nm. At pH 10.2 the extinction coefficient at 294 nm is 474.

When the substrate concentration exceeded the K_m value initial rates were obtained directly from the recorder traces; at substrate concentrations below K_m the recorder traces were pseudo-first order and rate constants were obtained from plots of $\log(A_\infty - A_t)$ against time.

Conjugation of Enzyme with Polymer.—The enzyme (100 mg) in water (5 ml) was mixed with polymer (50 mg) in water (5 ml) and the pH of the solution raised to 6.8; 1-ethyl-3-(3-*NN*-dimethylaminopropyl)carbodi-imide HCl (EDC) (20 mg) was added and the mixture stirred overnight at 4 °C. Addition of the enzyme to the polymer solution produced turbidity, owing to ionic complex formation, which disappeared as the pH was raised. During the time period the carboxy groups reacted completely with the polymer and any isourea or anhydride intermediate coupled with the enzyme or was hydrolysed.[11] The urea by-product and unbound enzyme were removed by ultrafiltration on Amicon filters with 30 000 m. wt. cut-off. The integrity of the enzyme–polymer conjugate was demonstrated by chromatography on an analytical Sephadex G25 column.

α-Chymotrypsin was coupled to CM-Sephadex (C-50) beads by essentially the same procedure as above except that unbound material was removed by centrifugation (MSE-Angle centrifuge).

The enzyme conjugated covalently to polymer-1 was tested for stability by incubating at pH 7.72 with tris(hydroxymethyl)-aminomethane (Tris) buffer at 0.1M ionic strength. The enzyme was assayed at intervals with BTEE at pH 7.72; after 1, 2, and 3 h the enzyme polymer conjugate had lost 11, 49, and 76% of the original activity compared with 66, 79, and 91% for the native enzyme in a control experiment.

Proof of Covalent Binding.—The enzyme–polymer conjugate was subjected to ultrafiltration with an Amicon Centricon filter with 30 000 m. wt. cut-off. Control experiments are carried out to demonstrate that the filters pass chymotrypsin; the effluent and supernatant are tested for activity with BTEE at pH 7.8.

Structural Measurements.—The dry diameters of the polymers were determined by transmission electron microscopy with uranyl acetate-stained materials on a Philips 410 electron microscope. The average diameters of the dry and wet CM-Sephadex were measured by standard microscopy; we are grateful to Mr. R. Newsam for help with these measurements. Wet diameters of polymer-1 under a variety of conditions of pH were measured with a Malvern Autoanalyser II; we are grateful to Dr. K. Salisbury for permission to use this instrument.

The polymers were titrated with standard alkali to estimate the carboxylic acid content; a Radiometer pH-titration set comprising REC Servograph, REA Titratigraph, pH-meter PHM 26, Titrator TTT 60, and Autoburette ABU 11 was employed.

Results

Polymer Structure.—Polymer-1 was essentially monodisperse with diameter 475 Å in the dry state. The wet diameter of polymer-1 was relatively constant above pH 9 with an average of 2 260 Å. Below pH 9 the apparent size increased and the polymer was less monodisperse (Table 1); this effect is due to coagulation of the polymer as the carboxylate ions are protonated at lower pH values. Probably the individual particles making up the larger lumps have the same diameter as at high pH giving overall the same surface area.

Table 1. Michaelis–Menten parameters for the substrate BTEE with native and immobilised chymotrypsin[a,b,f]

	Polymer–1–EDC conjugate			Native enzyme	
	k_{cat}/s^{-1} [c]	$10^5 K_m/M$ [d]	$d_{w.n}/nm$ [e]	k_{cat}/s^{-1} [c]	$10^5 K_m/M$ [d]
5.03	3.1	5.6		1.3	2.1
5.57	3			5.1	1.4
6.00			1 230 (0.48)		
6.58	34	2.0			
6.59				27	2.5
7.00			760 (0.48)		
7.74	69	2.0			
7.76				68	1.3
8.00			400 (0.31)		
8.40	69	2.3			
8.46				66	2.2
8.91				88	2.8
9.00			224 (0.28)		
9.01	61	2.6			
10.00			224 (0.28)		
10.16				71	39
10.20	63	37			
11.00			217 (0.24)		
12.20			238 (0.23)		
	CM-Sephadex–EDC conjugate			Native enzyme	
7.8	0.22			64	

[a] Ionic strength maintained at 0.1M with KCl, 25 °C. [b] Errors on the derived parameters are $< \pm 5\%$. Each parameter is the result of three different determinations. [c] Values of k_{cat} determined from initial rates with [BTEE] between 0.2 and 1mM for pH up to 9.01 and between 1 and 10mM for higher pH. [d] Values of K_m were determined from k_{cat}/K_m and k_{cat}; the values of k_{cat}/K_m were from pseudo-first-order rate constants with [BTEE] $< 2 \times 10^{-5}$M at pH up to 9.01 and 0.1mM for higher pH. [e] The volume-averaged size is $d_{w.n}$; the data were obtained with the Malvern Autoanalyser II and the Malvern polydispersity index is given in parentheses. [f] Active-site concentrations of enzyme were of the order of 5×10^{-6}M.

Hydrogen ion titration showed that the polymer-1 possesses 3.87×10^{-7} mol of equivalent acid (presumably carboxylic acid) per mg of solid. Based on a 15% molar composition of the feed there is thus 31% incorporation of the acrylic acid into the microgel; this is reasonable as the acrylic acid would not partition efficiently from water into the relatively non-polar growing bead of microgel polymer. The molecular weight of the polymer bead (excluding water) can be calculated from the dry diameter assuming a reasonable density of unity to be 3.38×10^7 and the number of carboxylate groups per bead as 1.31×10^4. The enzyme conjugate with polymer-1 used throughout the work was the same and had 940 active sites of enzyme per polymer particle.

Average diameters of the dry and wet CM-Sephadex were 5×10^{-3} and 1.3×10^{-2} cm respectively with a bound-enzyme content of 2.85×10^{11} active sites per particle.

Polymer-1 has one chymotrypsin molecule per 754 $Å^2$ of dry particle surface and per 1.7 10^4 $Å^2$ wet particle surface; the corresponding densities of carboxylate are 1 per 54 and 1 per 1 220 $Å^2$, respectively. In the conjugate with CM-Sephadex the total number of enzyme molecules per particle divided by the surface area is 1 per 2.8 $Å^2$ dry surface and 1 per 54 $Å^2$ wet surface.

Enzyme Activity.—The hydrolysis of low concentrations of BTEE catalysed by chymotrypsin exhibited pseudo-first-order kinetics. The rate constants were not altered by addition of a

Table 2. Values of k_{cat} for the chymotrypsin-catalysed hydrolysis of 4-nitrophenyl acetate [a,e]

pH	$10^3 k_{cat}$/s^{-1} [d] Native enzyme	Polymer-1 conjugate [b]
6.91	2.7	2.6
7.27	4.7	4.2
7.71	6.7	6.2
7.80	7.7	4.4 [c]
8.22	7.8	7.3
8.64		6.8
8.70	7.4	
8.97		5.1
9.04	6.0	
9.23		2.4
9.3	4.1	

[a] Ionic strength maintained at 0.1M with KCl, 25 °C. [b] Enzyme–polymer-1 ionic conjugate. [c] Enzyme–CM-Sephadex–EDC conjugate. [d] Each parameter is derived from three initial rates at varying substrate concentrations (4—10 × 10^{-5}M); the rates were not dependent on substrate concentration (thus $K_m < 4 \times 10^{-5}$M in each case). Errors in the derived parameters are $\leqslant \pm 5\%$. [e] Enzyme active-site concentrations were between 2 and 10 × 10^{-6}M.

second portion of substrate at the same concentration after all the first batch had been hydrolysed; this indicates that, provided the substrate concentration does not exceed this level, the product is not acting as an inhibitor. The same result holds over the whole of the pH range studied. The pseudo-first-order rate constant is thus given by $k_{obs} = [E_0]k_{cat}/K_m$. The initial rate at high substrate concentration is given by $[E_0]k_{cat}$ and this was demonstrated by reducing the concentration of substrate when the same initial rate was observed. The values of k_{cat}/K_m and k_{cat} were obtained by division of the first-order rate constant and initial rate respectively by the enzyme concentration (E_0) obtained from the active-site titration with 4-nitrophenyl acetate.

The Michaelis–Menten data for BTEE and 4-nitrophenyl acetate are displayed in Tables 1 and 2 for native enzyme, enzyme conjugated by EDC with polymer-1 and CM-Sephadex, and enzyme conjugated ionically with polymer-1. The data for the native enzyme agree with those determined by Berezin *et al.* (k_{cat} 85.9 s^{-1}; K_m 0.022mM at pH 7.8).[12]

The EDC-conjugate between enzyme and polymer-1 was coagulated by reducing the pH of the solution to 4.0; centrifugation followed by re-solution of the solid in fresh buffer at pH 7.8 gave a conjugate which had not lost its activity against BTEE. Freeze drying after cooling the enzyme–polymer-1 EDC-conjugate with a dichlorodifluoromethane bath cooled in liquid nitrogen gave fluffy white material which re-dissolved completely in water and suffered no loss of activity.

Coupling.—The yield in coupling chymotrypsin with polymer-1 with EDC was 80%.

Both polymers formed ionic complexes with the enzyme which were broken as the pH was raised to the operating pH of the enzyme. The yield of conjugated enzyme in coupling with CM-Sephadex in the presence of EDC was 18%.

At pH 7.0 and 9.2 Centricon ultra filters (m. wt. cut-off 30 000) did not pass either of the two microgel polymers but did pass native chymotrypsin. Of the original total activity, 95% was retained in the supernatant when the polymer-1 conjugate from EDC coupling was filtered at pH 9.2. The enzyme is slightly negative at pH 9.2 and therefore any non-conjugated enzyme will not bind ionically to the polymer. The EDC-conjugate between polymer-2 and the enzyme retained only 44% of the total activity in the supernatant and 33% was passed at pH 9.2; the 33% balance of activity was probably adsorbed on the membrane. The ionic conjugates between enzyme and the polymers passed all the activity at pH 9.2; at pH 7.0 no activities passed through the filter with these conjugates indicating significant binding between enzyme and polymer at pH 7.0.

Chromatographic analysis on Sephadex G25 at pH 9.2 indicated a single peak for polymer-1 EDC-conjugate.

Discussion

Polymer Conjugation.—The ultra-filtration and chromatography results demonstrate that covalent coupling occurs between chymotrypsin and polymer-1 in the presence of EDC. Polymer-2 couples inefficiently with chymotrypsin in the presence of EDC possibly due to steric hindrance at the methacrylic acid units retarding amide formation to an amino function on the enzyme. The carboxy groups of polymer-2 are attached to tertiary carbon and the methacrylic acid monomer would also tend to incorporate in the bulk of the polymer rather than at the surface; the acrylic acid would tend to incorporate at the surface in polymer-1. Inaccessibility is well known in solid-phase immobilisation where 'spacer' molecules are often necessary to enable efficient conjugation to take place.[1] The chymotrypsin is conjugated to polymer-1 at a level of one per 1.7 × 10^4 Å^2 surface calculated on the wet particle diameter where the occupancy of carboxy groups is one per 1 230 Å^2. About 9% of the wet microgel surface is covered by the enzyme as judged from the dimensions of chymotrypsin.[13] Suen and Morawetz[5b] obtained a 25% coverage of microgel with bovine serum albumin. It is most likely that the chymotrypsins reside on the microgel surface but the carboxys probably occupy to a depth as they will have been incorporated into the polymer at both early and late stages of the polymerisation. The coverage of the CM-Sephadex by enzyme is nominally at one molecule per 6.1 Å^2 of wet particle surface; this loading figure indicates that the enzyme must be conjugated to a depth.

The non-covalent conjugation of enzyme to polymer indicates that electrostatic coupling is not an efficient mode as it is broken readily by competition with small cations.

Kinetics.—The value of k_{cat} and K_m are almost the same for the native as for the polymer-1 EDC conjugate over a pH range; this is consistent with the polymer not interfering with the catalytic properties of the enzyme. Since at relatively high pH (9—10) the substrate becomes negatively charged the absence of any effect of the negatively charged microgel is conclusive evidence of no interaction between enzyme and microgel. Since only 9% of the microgel surface is utilised the absence of effects cannot be due to complete masking of the particle by enzyme. The observed properties of the enzyme are consistent with electrical neutrality at the microgel surface where the high negative charge at alkaline pH (1.31 × 10^4) is balanced by small bound cations. At high pH, where the substrate BTEE is ionised, the K_m is similar to that in the free enzyme. A highly negative microgel should repel the ionised substrate leading to much higher K_m values than in the native state.

Enzymes attached to solid ionic phases often exhibit pK perturbation due to the conjugated enzyme interacting with the electrostatic potential of the polymer.[14] Polymer-1 shows no perturbation despite the very large potential electrostatic effects due to the large charge and relatively small diameter of the particle. The perturbation of enzyme activity in a conjugate of chymotrypsin with a poly(methyl methacrylate–co-acrylic acid) latex found by Bahadur *et al.*[4] could be explained by a fluxional three-dimensional structure for these lattices where the enzyme could become buried in the polymer architecture. There was no cross-linking agent in the polymer feed so that at the pH values

concerned the polymer could be extended to envelope the enzyme with a negatively charged chain. The cross-linking in polymer-1 prevents significant extension of the particle (Table 1 indicates no increase in diameter as the pH is increased; the increase in apparent diameter at low pH is due to coagulation).

The values for k_{cat} for the hydrolysis of 4-nitrophenyl acetate in the presence of the polymer-1 ionic conjugate are almost identical with those of the native enzyme at low pH (Table 2). Above pH 9 the enzyme polymer complex becomes less active than the native. We believe this is due to the enzyme–polymer-1 conjugate breaking down due to the enzyme becoming negatively charged; the ester would then be adsorbed by the polymer and some of it would not be available for reaction with the enzyme. The absorption phenomenon has recently been probed by us;[6b] when the enzyme is bound to the polymer at low pH the partitioning does not seem to affect the kinetics.

The microgel offers a little stabilisation to the EDC-conjugated enzyme compared with that of the native enzyme. The stabilisation is relatively modest compared with that offered by other types of polymer support[2a,b] and with that for chymotrypsin attached to linear polyacrylate polymers.[4] The decay of chymotrypsin activity at pH 7—8 is caused by autolysis whereby the enzyme catalyses the hydrolysis of another enzyme molecule; this probably occurs more readily with the microgel-bound enzyme than with enzyme attached to for example solid phases because it will be much more accessible to an enzyme bound to another microgel. We deduce from this that the microgel-bound chymotrypsin is active against polymeric substrates; Seitz and Pauly[5a] showed that proteinase-K bound to a microgel possessed *ca.* 40% of the activity of the native protease against the polymeric substrate azocasein.

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