The Reaction of an α -Aza-Amino Acid Derivative with Chymotrypsin and Its Use as a Ligand for Covalent Affinity Purification

By S. A. BARKER, C. J. GRAY, J. C. IRESON and R. C. PARKER
 Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

and J. V. McLAREN
 Department of Physical Sciences, The Polytechnic, Wolverhampton WV1 1LY, U.K.

(Received 21 November 1973)

1. Chymotrypsin is inactivated by *N*-acetyl- α -azaphenylalanine phenyl ester (phenyl *N*²-acetyl-*N*¹-benzylcarbазate) in a stoichiometric reaction. 2. The inactivation is reversible spontaneously (first-order rate constant is $1.2 \times 10^{-4} \text{ s}^{-1}$) and accelerated by the presence of hydroxylamine. 3. Polymers based on polyacrylamide and carrying ligands containing the α -azaphenylalanine phenyl ester group were prepared. 4. Chymotrypsin reacts with these polymers and is removed by them from solution. Trypsin reacts less rapidly. 5. Chymotrypsin is slowly released from the polymer spontaneously and more rapidly on treatment with hydroxylamine. 6. The reaction of trypsin can be inhibited by competitive inhibitors. 7. Chymotrypsin was separated from trypsin by the selective bonding of chymotrypsin on to and its subsequent liberation from one of the polymers described.

Affinity chromatography, which is based on the reversible association between a protein (usually an enzyme) and a specific ligand covalently attached to a water-insoluble matrix, is now a well-established method for the purification of such proteins (Cuatrecasas & Anfinsen, 1971). Ashani & Wilson (1972) extended this concept to the use of a specific ligand whose interaction with the protein resulted in the formation of a covalent link. Their work was based on the irreversible inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by organophosphate and similar esters containing good leaving groups. The activity of the enzyme, inhibited by such compounds, can be restored subsequently by a variety of nucleophiles (Wilson & Ginsburg, 1955; Davies & Green, 1955; Hobbiger *et al.*, 1958; Poziomek *et al.*, 1958; Heilbronn, 1964; Wilson, 1951). Ashani & Wilson (1972) showed that acetylcholinesterase could be trapped by a solid matrix carrying 2-aminoethyl-*p*-nitrophenyl methyl phosphonate groups, and the enzyme recovered by the use of certain reactivators.

Kurtz & Niemann (1961*a*) studied the effect on chymotrypsin (EC 3.4.21.1) of ethyl *N*²-acetyl-*N*¹-benzylcarbазate (I, R = C₂H₅), the α -aza analogue of the chymotrypsin substrate *N*-acetyl-L-phenylalanine ethyl ester. From their results they concluded that the analogue was a poor competitive inhibitor of the enzyme and was not a substrate.

Elmore & Smyth (1968) reported the use of another derivative of the analogue, the *p*-nitrophenyl ester (I, R = C₆H₄NO₂), as a reagent for determining the absolute molarity of active chymotrypsin in solution. This process involves the rapid stoichiometric acylation of the active-site serine residue with the liberation of 1 mol of *p*-nitrophenol/mol of enzyme. The liberated *p*-nitrophenol was determined spectrophotometrically. An obvious consequence of the process is that the enzyme is irreversibly inhibited by the reagent since the deacylation step must be very slow if the titration is to be valid. The acyl-enzyme in this case involves a carbamoyl-serine, in contrast with the case for a normal substrate where a carboxyl ester is present.

A similar carbamoyl-serine intermediate was detected by Erlanger *et al.* (1966), who showed that diphenylcarbамoyl chloride was an efficient active-site-directed irreversible inhibitor of chymotrypsin.

The reaction of certain esterases with carbamate esters is clearly analogous. Some carbamates are very powerful inhibitors of acetylcholinesterase (Aldridge, 1969), and Wilson *et al.* (1961) showed that a carbamoyl-enzyme is produced and that the deacylation is very slow compared with that for a normal substrate.

In the present paper we report that the phenyl ester of the analogue (I, R = C₆H₅) also rapidly inactivates chymotrypsin and, more importantly, that the acyl-enzyme produced, which is the same as that produced by the reagent of Elmore & Smyth (1968), undergoes deacylation at a significant rate at 37°C. In addition, the deacylation step, which is very slow at 4°C, is accelerated in the presence of hydroxylamine.

Since the inactivation of chymotrypsin by the aza analogue is rapidly reversed in this way by the use of hydroxylamine, the analogue appeared to be worthy of investigation as a ligand for a covalent affinity material for chymotrypsin.

Experimental

Materials and methods

α -Chymotrypsin [3 $\times$ crystallized, and prepared free of autolysis products and contaminants by the method of Yapel *et al.* (1966)], trypsin (EC 3.4.21.4) (type III, 2 $\times$ crystallized), chymotrypsinogen and pepsin (EC 3.4.23.1, 2 $\times$ crystallized) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Human serum albumin for protein standard curves was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. T.l.c. was carried out by using Merck 'Kieselgel G nach Stahl' and the following solvent systems: A, butanol-acetic acid-water-pyridine (7:1:10:2, by vol.); B, chloroform-ethanol (19:1, v/v); components were detected by using iodine vapour, ninhydrin or the chlorine spray of Pan & Dutcher (1956).

All solvents were distilled before use. Proteinase activities of chymotrypsin and trypsin preparations were determined by the casein method of Laskowski (1955), modified to allow the concentration of hydrolysis products in the supernatant after precipitation by trichloroacetic acid to be determined by using the Folin-Ciocalteu reagent in a manner similar to that described by Anson (1939). It was established for all enzyme assays that over the range required the method was linear with respect to both time and enzyme concentration. Protein concentrations were determined by the method of Lowry *et al.* (1951). Specific assays for chymotrypsin and trypsin were carried out by the method of Schwert & Takenaka (1955), with the substrates acetyl-L-tyrosine ethyl ester and benzoyl-L-arginine ethyl ester respectively.

Synthesis of analogue derivatives

N-Acetyl- α -azaphenylalanine phenyl ester (I, R = C₆H₅). This was prepared by treatment of *N*-acetyl-*N'*-benzylhydrazine (Kurtz & Niemann, 1961b) (1.0g) in ethyl acetate (150ml) containing triethylamine (0.63g) with a solution of phenyl chloroformate (0.95g) in ethyl acetate (100ml). The mixture was stirred at 0°C for 1h, washed with 0.1M-Na₂CO₃, 0.05M-HCl and water, dried and then evaporated to dryness. The residue was crystallized from ethyl acetate-hexane to give colourless needles, m.p. 109–110°C (Found: C, 67.6; H, 5.8; N, 10.2. C₁₆H₁₆N₂O₃ requires C, 67.5; H, 5.6; N, 9.9%). The i.r. and n.m.r. spectra were consistent with the expected structure, as were the spectra of all the other compounds here described.

Boc^{*}- α -azaphenylalanine phenyl ester (phenyl *N*²-*t*-butyloxycarbonyl-*N'*¹-benzylcarbазate). Benzaldehyde (0.77ml) in ethanol (10ml) was added to a stirred solution of *t*-butylcarbазate (1.0g) in ethanol (10ml). After 1h, water (40ml) was added and the product, *N*-*t*-butyloxycarbonyl-*N'*-benzylidene hydrazide, was filtered off and crystallized from aq. ethanol (1.39g), m.p. 189–191°C (Found: C, 65.2; H, 7.5; N, 12.4. C₁₂H₁₆N₂O₂ requires C, 65.2; H, 7.5; N, 12.7%) [t.l.c.: iodine-positive; *R*_{F(B)} 0.84].

Hydrogenation of this benzylidene derivative, in ethanol at atmospheric pressure, in the presence of 10% palladium on charcoal gave *N*-butyloxycarbonyl-*N'*-benzylhydrazide as an oil which was homogeneous on t.l.c. [iodine-positive; *R*_{F(B)} 0.73].

This hydrazide (3.75g) in ethyl acetate (200ml) containing triethylamine (2.45ml) at 0°C was treated with phenyl chloroformate (2.7g), added slowly. The mixture was washed with dilute NaHCO₃, dilute HCl and water, dried and evaporated to give *Boc*- α -azaphenylalanine phenyl ester as a solid residue which was recrystallized from light petroleum (b.p. 60–80°C)-ethyl acetate (2.83g), m.p. 108–110°C [t.l.c.: iodine-positive; *R*_{F(B)} 0.66] (Found: C, 66.6; H, 6.7; N, 8.5. C₁₉H₂₂N₂O₄ requires C, 66.7; H, 6.4; N, 8.2%).

α -Azaphenylalanine phenyl ester (phenyl *N*¹-benzylcarbазate). *Boc*- α -azaphenylalanine phenyl ester (1.7g) was dissolved in trifluoroacetic acid (12ml) and the mixture left at room temperature for 1h. Most of the acid was removed by evaporation at 40°C. Water (40ml) was added and the solution adjusted to pH 8 with NaHCO₃. It was then extracted with dichloromethane and the extracts washed with dilute NaHCO₃, dilute HCl and water, dried and evaporated to give α -azaphenylalanine phenyl ester as an oil (1.15g) [t.l.c.: iodine-positive; *R*_{F(B)} 0.73]. This was characterized as its *N*-benzoyl derivative (phenyl *N*²-benzoyl-*N'*¹-benzylcarbазate), m.p. 132–134°C (Found: C, 72.9; H, 5.1; N, 8.4. C₂₁H₁₈N₂O₃ requires C, 72.8; H, 5.2; N, 8.1%).

N-Acryloyl-6-aminohexanoic acid. Acryloyl chloride (9.6ml) was added over 1.5h, with stirring, to a solution of 6-aminohexanoic acid (12.5g) in 0.1M-NaHCO₃ (100ml) at 0°C. During the addition, 5M-NaOH was added to maintain the pH at 7.5–8.5. The mixture was then acidified to pH 2.0 with conc. HCl and kept at 0°C until the product had precipitated. It was filtered off, washed with water and recrystallized from water (5.1g), m.p. 77–79°C (Found: C, 58.3; H, 8.0; N, 7.7. C₉H₁₅NO₃ requires C, 58.4; H, 8.1; N, 7.6%) [t.l.c.: ninhydrin-negative; chlorine-positive; *R*_{F(A)} 0.67].

N-Acryloyl-4-aminobutyric acid. This was prepared by a method similar to that described for the amino-hexanoic acid analogue. The product was crystallized

* Abbreviation: Boc, *t*-butyloxycarbonyl.

from water, m.p. 96–98°C (Found: C, 53.4; H, 7.3; N, 8.8. $C_7H_{11}NO_3$ requires C, 53.5; H, 7.0; N, 8.9%) [t.l.c: ninhydrin-negative; chlorine-positive; $R_{F(A)}$ 0.70.]

(*N*-Acryloyl-6-aminohexanoyl)- α -azaphenylalanine phenyl ester (phenyl N^2 -(*N*-acryloyl-6-aminohexanoyl)- N^1 -benzylcarbazate). Dicyclohexylcarbodi-imide (1.29g) was added to a cooled, stirred solution of *N*-acryloyl-6-aminohexanoic acid (1.05g) and α -azaphenylalanine phenyl ester (1.37g) in dioxan (100ml) and dichloromethane (80ml). After being stirred for 1 h at 0°C and for 14 h at 20°C, the mixture was filtered and dichloromethane (200ml) added to the evaporated filtrate. The resultant solution was then washed and dried, and on evaporation yielded an oil (2.26g). The oil was chromatographed on a column of silica gel (Merck, 80g) in chloroform. Elution with chloroform gave azaphenylalanine phenyl ester and another component. Elution with 2% (v/v) ethanol in chloroform gave the desired product (1.54g) which was recrystallized from chloroform-hexane, m.p. 88–90°C (Found: C, 67.3; H, 6.6; N, 10.2. $C_{23}H_{27}N_3O_4$ requires C, 67.6; H, 6.6; N, 10.3%) [t.l.c: iodine-positive; $R_{F(B)}$ 0.38].

(*N*-Acryloyl-4-aminobutyl)- α -azaphenylalanine phenyl ester (phenyl N^2 -(*N*-acryloyl-4-aminobutyl)- N^1 -benzylcarbazate). This was prepared by a method similar to that described for the 6-aminohexanoyl analogue; m.p. 95°C (decomp.) (Found: C, 66.3; H, 6.3; N, 10.8. $C_{21}H_{23}N_3O_4$ requires C, 66.2; H, 6.0; N, 11.0%) [t.l.c: iodine-positive; $R_{F(B)}$ 0.30].

Preparation of polymers

Polymer I. A solution of (*N*-acryloyl-6-aminohexanoyl)- α -azaphenylalanine phenyl ester (0.215g) in dioxan (7ml) was mixed with a solution of acrylamide (1.80g) and *NN'*-methylenebisacrylamide (0.19g) in water (20ml) and the mixture purged with N_2 for 30min. Traces of potassium persulphate and riboflavin were then added and, while N_2 was passed over the surface, the mixture was irradiated for 45 min with a Phillips no. 2 Photoflood lamp. The gel produced was broken up, washed well with water and then with ethanol. The solid was dried *in vacuo* over P_2O_5 , ground in a mortar and sieved to give particles of 30–60 mesh (1.91g).

Polymer II. This was prepared in the same way as described above except that only 0.044g of (*N*-acryloyl-6-aminohexanoyl)- α -azaphenylalanine phenyl ester was incorporated into the polymer (yield 1.99g).

Polymer III. This was prepared as described above, but with (*N*-acryloyl-4-aminobutyl)- α -azaphenylalanine phenyl ester (0.215g) in place of the aminohexanoyl analogue (yield 1.96g).

Polymer IV. This is similar to polymer III except that only 0.044g of (*N*-acryloyl-4-aminobutyl)-

azaphenylalanine phenyl ester was incorporated into the polymer (yield 2.0g).

All polymers were kept *in vacuo* over P_2O_5 until used.

Chymotrypsin studies

Incubation of α -chymotrypsin with *N*-acetyl- α -azaphenylalanine phenyl ester. α -Chymotrypsin (0.064 μ mol) in 0.05M-sodium phosphate buffer, pH 7.0 (10ml), was incubated at 37°C with *N*-acetyl- α -azaphenylalanine phenyl ester (0.064 μ mol) in methanol (0.20ml) for several hours. The chymotryptic activity was determined periodically by removing samples (0.05ml) for assay by the casein method by using an incubation period of 5 min. A control experiment was conducted in which methanol only was added to the chymotrypsin solution.

A similar experiment was performed with 0.032 μ mol of analogue.

Preparation of *N*-acetyl- α -azaphenylalanyl-chymotrypsin. To α -chymotrypsin (1.5mg, 0.060 μ mol) in 0.05M-sodium phosphate buffer, pH 7.0 (1.0ml), was added a solution of *N*-acetyl- α -azaphenylalanine phenyl ester (0.135 μ mol) in methanol (0.04ml) and the mixture was incubated at 37°C for 20min. A sample (0.5ml) was chromatographed at 4°C on a column (1cm $\times$ 20cm) of Bio-Gel P-10 equilibrated with 0.05M-sodium phosphate buffer, pH 7.0. The protein fractions eluted were pooled and stored at 4°C. The specific activity was determined by the casein (Laskowski, 1955) and Lowry *et al.* (1951) assays.

A control preparation was obtained in the same way except that methanol only was used in place of analogue solution.

Reactivation at room temperature. Samples (approx. 1ml) of deactivated and control chymotrypsin were left at room temperature (approx. 20°C) for 16h. Enzyme activities were then determined and compared with the activities of comparable solutions that had been stored at 4°C.

Reactivation by hydroxylamine. To portions (2.0ml) of the deactivated and control chymotrypsin solutions were added portions of a solution of hydroxylamine in water (4.2M; 0.4 ml; previously adjusted to pH 7.0 with NaOH solution). After keeping at 4°C for 16h the solutions were dialysed against 0.05M-sodium phosphate buffer, pH 7.0, at 4°C. After dialysis, the specific activities of the solutions were determined and compared with the activities of comparable solutions that had not been treated with hydroxylamine.

Deactivation of α -chymotrypsin with *N*-acetyl- α -azaphenylalanine ethyl ester. To a solution of α -chymotrypsin (3.1 μ g/ml; 3.0ml containing 0.372nmol) in 0.05M-sodium phosphate buffer, pH 7.0, at 37°C, was added a solution of *N*-acetyl- α -azaphenylalanine ethyl ester (49.5mg/ml; 0.15ml containing 31.4 μ mol) in acetonitrile. The mixture

was incubated at 37°C and samples (0.25ml) were removed at intervals and assayed by the casein method with a 30 min incubation period. A control experiment was conducted with acetonitrile only in place of *N*-acetyl- α -azaphenylalanine ethyl ester solution.

Reaction with polymers

Reaction of chymotrypsin and trypsin with polymer I. Polymer I (6.0mg) was added to a solution of chymotrypsin (2.2mg) in 0.05M-sodium phosphate buffer (pH7.0, 5.0ml) and the mixture stirred at 25°C. Samples (0.025ml) were removed at intervals and assayed for enzymic activity and protein remaining in the solution by using the casein and Lowry *et al.* (1951) methods respectively. The results were compared with a control in which no polymer was added.

A similar experiment was carried out in which trypsin (2.2mg) was used in place of chymotrypsin, the only difference in method being that 0.01ml samples were removed for assay. Similar experiments were carried out in which chymotrypsinogen and pepsin were exposed to polymer I. In these cases, only the protein contents of the supernatants were measured.

Reaction of chymotrypsin with polymers I, II, III and IV. Polymers I, II, III and IV (6.0mg each) were stirred separately with chymotrypsin solutions (2.2mg in 0.05M-sodium phosphate buffer, pH7.0; 5.0ml) at 25°C. In each case samples (0.025ml) were removed at intervals and assayed for enzymic activity and protein content. A similar experiment was carried out with trypsin (2.2mg), 0.01ml samples being removed and assayed.

Liberation of chymotrypsin from polymer I. (a) Spontaneously. Polymer I (1.3mg) was stirred with chymotrypsin (1.08mg) in 0.05M-sodium phosphate buffer (pH7.0; 5.0ml) at 37°C. After 3h the supernatant was removed and the polymer washed with cold 0.05M-phosphate buffer (pH7.0; 8 $\times$ 5ml). The solid was then suspended in the same buffer (5.0ml) and the mixture stirred at 37°C. At intervals samples (0.05ml) of supernatant were removed and assayed for enzymic activity by the casein assay.

(b) By hydroxylamine. Polymer I (6.0mg) was stirred with chymotrypsin (2.22mg) in 0.05M-sodium phosphate buffer (pH7.0; 5.0ml) at 37°C for 1h. The supernatant was removed and assayed for enzymic activity and protein content. The solid was then washed with cold 0.05M-sodium phosphate buffer (pH7.0; 8 $\times$ 5ml). Two identical batches of material were prepared in this manner. One was treated with hydroxylamine solution (prepared from 0.7M-hydroxylamine hydrochloride adjusted to pH7.0 with NaOH solution; 5.0ml) at 4°C for 18h, the other with a 0.05M solution of phosphate buffer (pH7.0; 5.0ml) at 4°C for 18h. The mixtures were

centrifuged and the supernatants dialysed against the same phosphate buffer for 6h and the enzymic activity and protein content determined.

Effect of trypsin inhibitors on the reaction of trypsin and chymotrypsin with polymer I

A batch of polymer I (6.0mg) was stirred at 25°C with trypsin (2.2mg) in 0.05M-sodium phosphate buffer (pH7.0; 5ml). The solution was 0.005M in *n*-butylamine. At intervals, samples (10 μ l) were removed, diluted to 50 μ l and assayed for enzymic activity by the casein method. Three other batches of polymer I were treated in the same way except that the solutions were 0 or 0.002M in *n*-butylamine or 0.01M in *N*- α -benzoyl-L-arginine respectively.

Similar experiments were carried out by using chymotrypsin in place of trypsin.

Separation of trypsin and chymotrypsin by using polymer I. (a) Polymer I (6.0mg) was stirred at 25°C with a solution of trypsin (2.2mg) and chymotrypsin (2.2mg) in 0.05M-sodium phosphate buffer (pH7.0; 5.0ml). After 1h the mixture was centrifuged and the supernatant assayed for trypsin activity, chymotrypsin activity and protein content. (b) Polymer I (6.0mg) was stirred at 25°C with a solution of trypsin (2.2mg) and chymotrypsin (2.2mg) in 0.05M-sodium phosphate buffer (pH7.0; 5ml) which was also 0.01M in *N*- α -benzoyl-L-arginine. After 1h the solution was centrifuged and the supernatant was assayed for trypsin activity, chymotrypsin activity and protein content. The solid residue was then washed with the phosphate buffer and then stirred with a hydroxylamine solution (0.7M-hydroxylamine hydrochloride adjusted to pH7.0 with NaOH solution; 5.0ml) at 4°C for 18h. The mixture was then centrifuged and the supernatant dialysed against the phosphate buffer for 6h. It was then assayed for trypsin and chymotrypsin activity and for protein content.

Results

Fig. 1 shows how the activity of α -chymotrypsin varies with time on treatment with 1 molar equivalent and with 0.5 molar equivalent of *N*-acetyl- α -azaphenylalanine phenyl ester (I, R = C₆H₅) at pH7.0 at 37°C. Clearly the enzyme is rapidly inactivated by a reaction that is stoicheiometric. The deactivation is followed by a much slower reactivation, which must represent the deacylation step. It was possible to calculate a value for the first-order rate constants for the deacylation from a plot of log (inactive enzyme remaining) versus time, the slope being calculated by a least-squares procedure. The average value from the two curves was $1.2 \times 10^{-4} \text{ s}^{-1}$.

N-Acetyl- α -azaphenylalanyl-chymotrypsin is relatively stable at 4°C. A sample prepared by chromatography as described regained no significant activity

Fig. 1. Variation with time of activity of α -chymotrypsin treated with *N*-acetyl- α -azaphenylalanine phenyl ester

α -Chymotrypsin ($6.25\mu\text{M}$) was treated with 1 molar equivalent (●) and with 0.5 molar equivalent (○) of *N*-acetyl- α -azaphenylalanine phenyl ester at 37°C , pH 7.0. For the reactivation stages, the curves drawn correspond to the lines of best fit obtained from the plots of log (inactive enzyme remaining) versus time by a least-squares calculation.

Fig. 2. Variation with time of activity of α -chymotrypsin treated with *N*-acetyl- α -azaphenylalanine ethyl ester

α -Chymotrypsin ($0.118\mu\text{M}$) was treated with *N*-acetyl- α -azaphenylalanine ethyl ester (10mM) at 37°C , pH 7.0.

on storage for 16h at this temperature (Table 1). At room temperature (approx. 20°C) 33% activity was regained over 16h (Table 1).

Hydroxylamine increased the rate of reactivation at 4°C . Treatment for 16h caused regeneration to 87% of the original activity, whereas a sample not so treated showed no more than 5% (Table 1).

The effect of the ethyl ester (I, $\text{R} = \text{C}_2\text{H}_5$) on chymotrypsin was also studied. α -Chymotrypsin was treated with a large excess of this ethyl ester and the reaction followed for any loss of activity. The results (Fig. 2) indicated that there was a gradual, but significant, loss when the concentrations of ester and enzyme were 10mM and $0.118\mu\text{M}$ respectively (based on mol.wt. of 25000; Eck & Dayhoff, 1966).

The results represented in Fig. 3 indicated that chymotrypsin was rapidly removed from solution by polymer I. Assays of enzymic activity and protein content in the supernatant solution indicated that although virtually all the detectable enzymic activity had disappeared only just over 80% of the protein

Fig. 3. Reaction of α -chymotrypsin and trypsin with polymer I

The reaction was carried out at 25°C , pH 7.0. Other experimental details are given in the Experimental section. ○, Chymotrypsin, enzymic activity; ●, chymotrypsin, protein content; Δ, trypsin, enzymic activity; ▲, trypsin, protein content.

Table 1. Preparation and reactivation of *N*-acetyl- α -azaphenylalanine-chymotrypsin

Experimental details are given in the Experimental section.

Chymotrypsin preparation		Specific activity relative to chymotrypsin stored at 4°C (%)
Experiment	Description of sample	
Preparation of acyl-chymotrypsin	(a) Control; activity after column chromatography	100
	(b) Preparation; activity after column chromatography	4
Stability of acyl-chymotrypsin	(c) Control; stored at 4°C	100
	(d) Preparation (b); stored at 4°C	3
	(e) Control; stored at room temperature (16h)	90
	(f) Preparation (b); stored at room temperature (16h)	33
Treatment of acyl-chymotrypsin with hydroxylamine	(g) Control; stored at 4°C	100
	(h) Preparation (b); stored at 4°C	5
	(i) Control; treated with NH_2OH as described	85
	(j) Preparation (b); treated with NH_2OH as described	87

had been taken up. Fig. 3 also shows that under the same conditions trypsin was taken up considerably more slowly. After 3h, when the supernatant had lost 76% of the enzymic activity, it had lost 47% of the protein.

Neither chymotrypsinogen nor pepsin reacted with the polymer under these conditions.

In Fig. 4 the reaction of chymotrypsin with each of the four polymers is shown. Fig. 4 shows the activity remaining in the supernatants. An essentially similar pattern was obtained when the protein contents of the supernatants were measured. The reaction of trypsin with the four polymers also gave similar patterns

when both protein content and enzymic activity were measured, although trypsin reacted much more slowly in each case than did chymotrypsin.

The results shown in Table 2 indicate that chymotrypsin can be liberated from the polymer after incorporation. Table 2(a) shows that a significant amount of chymotrypsin activity is released into the supernatant after 24h at 37°C and at pH7.0. Table 2(b) indicates that treatment with hydroxylamine at 4°C for 16h caused liberation of 63% of the original chymotrypsin activity.

Fig. 5 shows that the already slow uptake of trypsin by polymer I is slowed even further by the presence of

Fig. 4. Reaction of chymotrypsin with polymers I, II, III and IV

The reaction was carried out at 25°C, pH 7.0. Other experimental details are given in the Experimental section. ○, Polymer I; □, polymer III; ●, polymer II; ■, polymer IV; Δ, control, i.e. no polymer added.

Fig. 5. Reaction of trypsin with polymer I; effect of competitive inhibitors

The reaction was carried out at 25°C, pH 7.0. Other experimental details are given in the Experimental section. ○, No inhibitor present; □, 0.002M-butylamine; Δ, 0.005M-butylamine; ●, 0.01M-N-benzoyl-L-arginine.

Table 2. Liberation of chymotrypsin after attachment to polymer I

Experimental details are given in the Experimental section.

(a) Spontaneous liberation of enzyme at 37°C at pH7.0.

Time (h)	Chymotrypsin activity in supernatant (% of original activity)
0	0
1	7
2	10
24	20

(b) Attachment of chymotrypsin to polymer I and its release by treatment with hydroxylamine at pH7.0, at 4°C for 18h.

	Chymotrypsin in supernatant after exposure to polymer I		Chymotrypsin in supernatant after release	
	Protein (% of original)	Chymotrypsin activity (% of original)	Protein (% of original)	Chymotrypsin activity (% of original)
Batch I	37.4	9.2	0*	0*
Batch II	37.4	10.0	54.5†	63†

* Batch I was treated with buffer only at 4°C.

† Batch II was treated with NH_2OH as described.

Table 3. Separation of chymotrypsin and trypsin by using polymer I

Experimental details are given in the Experimental section. n.d., not detectable.

Nature of experiment	Detected in supernatant		
	Trypsin activity (% of original)	Chymotrypsin activity (% of original)	Protein (mg) (% of original)
(a) Attachment to polymer I; no inhibitor present	60.0	n.d.	2.33 (53)
(b) Attachment to polymer I; 0.01 M-benzoyl- arginine present	96.0	n.d.	2.67 (61)
Released into supernatant by treatment with NH ₂ OH	n.d.	76	1.43 (32.5)

compounds *n*-butylamine and *N*- α -benzoylarginine, which are known competitive inhibitors of this enzyme.

Table 3 summarizes the results of experiments to confirm that chymotrypsin could be selectively removed from a solution in the presence of trypsin. In the first case, in the absence of a trypsin inhibitor the polymer took up all of the chymotrypsin present and 40% of the trypsin. In the presence of 0.01 M-benzoylarginine the removal of trypsin from solution is almost totally prevented whereas the chymotrypsin was completely taken up. No trypsin activity was subsequently liberated from the solid by the treatment with hydroxylamine, whereas the chymotrypsin activity detected represented 76% of the original used. Since only 32.5% of the total protein used initially was recovered this represents an enhancement of the specific activity of the chymotrypsin.

Discussion

In view of the work described by Elmore & Smyth (1968) the stoichiometric inactivation of chymotrypsin by *N*-acetyl- α -azaphenylalanine phenyl ester is not unexpected, although it is noteworthy that the phenyl ester does provide a sufficiently good leaving group for the acylation process to occur rapidly. Extrapolation of the curves in Fig. 1 to zero time suggests that 0.5 molar equivalent of analogue produced more than 50% inhibition, indicating that the chymotrypsin contained a certain amount (possibly 20%) of inactive protein. This conclusion is supported by results described later in this paper.

The subsequent spontaneous reactivation, which almost certainly represents the deacylation process, was, however, not expected. The first-order rate constant for the deacylation, which was calculated as $1.2 \times 10^{-4} \text{ s}^{-1}$, is significant, although it is low enough to confirm that deacylation is negligible under the conditions described by Elmore & Smyth (1968) for the titration experiment.

It is worth noting for comparison that the rate constants for the deacylation at 25°C of methyl-

carbamoyl- and dimethylcarbamoyl-acetylcholinesterase reported by Wilson *et al.* (1961) are 3.0×10^{-4} and $4.3 \times 10^{-4} \text{ s}^{-1}$ respectively.

By contrast, the rate constants for the deacylation of acyl-enzymes derived from normal α -chymotrypsin substrates are approx. 10^6 times that for *N*-acetyl- α -azaphenylalanyl-chymotrypsin. For example, at 25°C at pH 7.0, Zerner *et al.* (1964) found that the rate of deacylation of *N*-acetyl-L-phenylalanyl-chymotrypsin was 72 s^{-1} .

At 4°C deacylation is very slow and the α -aza-acyl-chymotrypsin can be stored at this temperature for 16 h without detectable increase in activity. At room temperature some significant regeneration of activity was observed over 16 h.

Both Wilson *et al.* (1961) with carbamoyl derivatives of acetylcholinesterase and Erlanger *et al.* (1966) with diphenylcarbamoyl-chymotrypsin have demonstrated that in the presence of hydroxylamine the reactivation is accelerated. We have confirmed that hydroxylamine increases the rate of reactivation of *N*-acetyl- α -azaphenylalanyl-chymotrypsin. Treatment with hydroxylamine for 16 h at 4°C resulted in recovery of 87% of the original activity compared with a sample not treated, which showed no more than 5% activity. Whether the deacylation is brought about by a direct nucleophilic attack by the hydroxylamine on the acyl-enzyme resulting in the formation of a hydroxamic acid derivative is not known.

The *p*-nitrophenyl ester described by Elmore & Smyth (1968), the diphenylcarbamoyl chloride of Erlanger *et al.* (1966) and the phenyl ester described in the present paper all possess good leaving groups, so that the acylation step takes place rapidly. The ethyl ester reported by Kurtz & Niemann (1961*a*) possesses a much poorer leaving group. The acylation process, if it occurred at all, would therefore be expected to be very slow in this case. Our results (Fig. 2) indicate that acylation does occur under the conditions described, although very slowly. Extrapolation of the curve back to zero time, giving an intercept of about 78%, suggests that a degree of competitive inhibition might be occurring, in some

agreement with Kurtz & Niemann (1961a). From the rate of deacylation of the *N*-acetyl- α -azaphenylalanyl-chymotrypsin, and assuming deacylation to be rate-limiting, it can be calculated that under the conditions used by Kurtz & Niemann (1961a) the maximum turnover rate would be so slow that hydrolysis to any significant extent is unlikely to be detected.

As a result of the work described here it is clear that the aza-analogue phenyl ester reacts with chymotrypsin to give a product in which the enzyme is covalently linked and from which the enzyme can be released by treatment with hydroxylamine. The analogue ester therefore fulfils the two essential criteria for a ligand in a covalent affinity material. Several such materials have been reported. Ashani & Wilson (1972) used a *p*-nitrophenylmethyl phosphonate as the active ligand for acetylcholinesterase, Blumberg & Strominger (1972) used bound 6-aminopenicillanic acid for penicillin-binding proteins and Brocklehurst *et al.* (1973) purified papain by using a material based on a 2-pyridyl disulphide. In each of these cases Sepharose provided the insoluble matrix. In our laboratories (Epton *et al.*, 1971; Barker *et al.*, 1970a,b, 1971) it has been established that highly satisfactory insoluble matrices for enzyme insolubilization can be obtained by the copolymerization of acrylamide with acryloylamido derivatives. Our covalent affinity materials were therefore prepared by the copolymerization of acrylamide and acryloylamido derivatives carrying the α -azaphenylalanine phenyl ester ligand. It is well established (Cuatrecasas & Anfinsen, 1971) that affinity materials are more effective if a long hydrocarbon chain is interposed between the polymer backbone and the ligand. For this reason we prepared the *N*-acryloyl-6-amino-hexanoyl- and *N*-acryloyl-4-aminobutyl derivatives of α -azaphenylalanine phenyl ester as monomers for incorporation into the copolymers. In each case cross-linking of the copolymer was achieved by incorporating about 10% of *NN'*-methylenebisacrylamide. Four copolymers were prepared, incorporating approx. 9.7% and 2.2% of the *N*-acryloyl-4-aminobutyl or *N*-acryloyl-6-amino-hexanoyl derivative of the α -aza analogue respectively.

Polymer I took up chymotrypsin rapidly; the supernatant after 1 h had lost virtually all the chymotrypsin activity (Fig. 3). Interestingly, at this stage and after longer periods of time, only a little more than 80% of the protein of the supernatant had been lost. This supports the result obtained with the soluble analogue, which indicated that the preparation contained up to 20% of inactive protein.

Trypsin, which differs in specificity from chymotrypsin but which functions by an essentially similar acyl-enzyme mechanism, was also removed from solution by polymer I. The rate of reaction in this case, however, was considerably slower (Fig. 3), indicating that the polymer is selective in its function

although not specific. This conclusion is in broad agreement with the observation of Elmore & Smyth (1968) that the reagent that they had devised for active-site titration reacted only slowly with trypsin.

The selectivity of the polymer's action was further emphasized by the observation that neither chymotrypsinogen nor pepsin reacted with the polymer.

All four polymers took up chymotrypsin effectively. The variation in the rates at which the enzyme was taken up indicated clearly that polymer I, which incorporated more of the *N*-acryloyl-6-amino-hexanoyl derivative than polymer II, functioned the more rapidly. In addition polymer I reacted with the enzyme slightly more rapidly than did polymer III, in which the ligand was separated from the polymer backbone by the smaller 4-aminobutyl moiety. The effect of the longer hydrocarbon chain on the rate of the reaction is even more noticeable when the two polymers II and IV, in which the concentrations of ligand are much smaller, are observed. In all cases, however, the enzyme had been removed from solution virtually completely in 3 h.

A similar pattern was observed when the reaction of trypsin with the polymers was investigated, the major difference being that in each case the uptake of enzyme was much slower than for chymotrypsin.

The value of a covalent affinity material is ultimately dependent on the subsequent release of the enzyme from the covalently bound complex. The deacylation of *N*-acetyl- α -azaphenylalanyl-chymotrypsin occurred spontaneously, if slowly, at 37°C and the results in Table 2(a) indicate that at this temperature, in water, the enzyme is released spontaneously from the solid material also. However, the release under these conditions is probably too slow for practical value (20% released after 24 h). Since hydroxylamine was shown to enhance the rate of deacylation of the enzyme inhibited by the soluble aza analogue, it is not surprising that it also enhanced the rate of liberation of the enzyme from the complex. Table 2(b) shows that 63% of the original activity was released by treatment with hydroxylamine for 16 h at 4°C. Interestingly, in this case the measured protein liberated represented only 54.5% of the original protein, indicating that the enzyme released possessed a higher specific activity than the original. An increase in specific activity of approx. 15% was thereby achieved.

Since polymer I did absorb trypsin at a significant rate, it could not on its own function as a specific covalent affinity absorbent for chymotrypsin. Some means had to be found which would prevent the uptake of trypsin by the polymer. Since by analogy it is assumed that trypsin reacts at its active site with the ligand of the polymer, normal competitive inhibitors of the enzyme were considered likely also to inhibit the reaction of the enzyme with the polymer. This proved to be the case. *n*-Butylamine (Inagami & Murachi,

1963) and *N*- α -benzoyl-L-arginine (Bechet *et al.*, 1966) are both effective competitive inhibitors of trypsin. They were also effective in inhibiting the reaction of trypsin with the polymer (Fig. 5) while having no effect on the uptake of chymotrypsin. These results therefore suggest that in the presence of trypsin inhibitors of this type, the reaction of chymotrypsin with polymer I could be highly selective.

This was confirmed by experiments in which the removal from solution of the enzymes from a mixture of equal quantities by weight of chymotrypsin and trypsin was studied. In the absence of a trypsin inhibitor both chymotrypsin (completely) and trypsin (partially) were taken up from the supernatant solution by polymer I. In the presence of 0.01 M-*N*- α -benzoyl-L-arginine, however, the amount of trypsin taken up was virtually insignificant. The presence of this inhibitor did not prevent the complete absorption of chymotrypsin. Subsequent treatment of the solid from this latter experiment with hydroxylamine liberated protein which possessed 76% of the original chymotrypsin activity, but no detectable trypsin activity. The protein liberated as determined by the Lowry *et al.* (1951) assay represented 32.5% of the total protein to which the polymer had been exposed, or 65% of the original chymotrypsin. The use of this polymer has thus achieved not only a complete separation of trypsin from chymotrypsin and chymotrypsin from trypsin, but also an increase in the specific activity of the chymotrypsin of 17%. This value is in broad agreement with the conclusion, drawn from the studies with the soluble inhibitor and also the original absorption of chymotrypsin on to polymer I, that the chymotrypsin preparation used contained up to 20% of inactive protein.

We thank Professor M. Stacey, C.B.E., F.R.S., for his interest in this work, the Science Research Council and Koch-Light Laboratories for a C.A.P.S. award (to J. C. I.) and the University of Birmingham for a Research Scholarship (to R.C.P.).

References

- Aldridge, W. N. (1969) *Brit. Med. Bull.* **25**, 236-239
 Anson, M. L. (1939) *J. Gen. Physiol.* **22**, 79-89
 Ashani, Y. & Wilson, I. B. (1972) *Biochim. Biophys. Acta* **276**, 317-322
 Barker, S. A., Somers, P. J., Epton, R. & McLaren, J. V. (1970a) *Carbohydr. Res.* **14**, 287-296
 Barker, S. A., Somers, P. J. & Epton, R. (1970b) *Carbohydr. Res.* **14**, 323-326
 Barker, S. A., Gray, C. J., Khoujah, A. M. & Law, C. M. J. (1971) *Birmingham Univ. Chem. Eng.* **22**, 74-80
 Bechet, J. J., Gardiennet, M. C. & Yon, J. (1966) *Biochim. Biophys. Acta* **122**, 101-115
 Blumberg, P. & Strominger, J. L. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 3751-3755
 Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. & Crook E. M. (1973) *Biochem. J.* **133**, 573-584
 Cuatrecasas, P. & Anfinsen, C. B. (1971) *Annu. Rev. Biochem.* **40**, 259-278
 Davies, D. R. & Green, A. L. (1955) *Discuss. Faraday Soc.* **20**, 269
 Eck, R. V. & Dayhoff, M. O. (1966) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, Maryland
 Elmore, D. T. & Smyth, J. J. (1968) *Biochem. J.* **107**, 103-107
 Epton, R., Thomas, T. H. & McLaren, J. V. (1971) *Biochem. J.* **123**, 21P-22P
 Erlanger, B. F., Cooper, A. G. & Cohen, W. (1966) *Biochemistry*, **5**, 190-196
 Heilbronn, E. (1964) *Acta Chem. Scand.* **18**, 2410
 Hobbiger, F., O'Sullivan, D. G. & Sadler, P. W. (1958) *Nature (London)* **182**, 1498-1499
 Inagami, T. & Murachi, T. (1963) *J. Biol. Chem.* **238**, PC1905-PC1907
 Kurtz, A. N. & Niemann, C. (1961a) *J. Amer. Chem. Soc.* **83**, 1879-1882
 Kurtz, A. N. & Niemann, C. (1961b) *J. Org. Chem.* **26**, 1843-1846
 Laskowski, M. (1955) *Methods Enzymol.* **2**, 26-36
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
 Pan, S. C. & Dutcher, J. D. (1956) *Anal. Chem.* **28**, 836-838
 Poziomek, E. J., Hackley, B. E. & Steinberg, G. M. (1958) *J. Org. Chem.* **23**, 714-717
 Schwert, G. W. & Takenaka, Y. (1955) *Biochim. Biophys. Acta* **16**, 570-575
 Wilson, I. B. (1951) *J. Biol. Chem.* **190**, 111-117
 Wilson, I. B. & Ginsburg, S. (1955) *Biochim. Biophys. Acta* **18**, 168-170
 Wilson, I. B., Harrison, M. A. & Ginsburg, S. (1961) *J. Biol. Chem.* **236**, 1498-1500
 Yapel, A., Moon, M., Rosenberg, A., Lumry, R. & Shiao, D. F. (1966) *J. Amer. Chem. Soc.* **88**, 2572-2584
 Zerner, B., Bond, R. P. M. & Bender, M. L. (1964) *J. Amer. Chem. Soc.* **86**, 3674-3679