A New Method for Determining the Absolute Molarity of Solutions of Trypsin and Chymotrypsin by using *p*-Nitrophenyl N^2 -Acetyl- N^1 -benzylcarbazate

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1. *p*-Nitrophenyl N^2 -acetyl- N^1 -benzylcarbazate (NPABC) was synthesized and shown to acylate α -chymotrypsin stoichiometrically; reaction at 25° occurs almost instantaneously at pH 7.04 and within 2 min. at pH 5.04 and there is no observable turnover during 10 min. 2. The absolute molarity of solutions of α -chymotrypsin can be determined by spectrophotometric measurement of the *p*-nitrophenol liberated during the acylation step; the results obtained at pH 5.04 and pH 7.04 agree with one another and with those determined by the method of Erlanger & Edel (1964). 3. Trypsin reacts stoichiometrically, but more slowly than α -chymotrypsin, with NPABC, and it, like chymotrypsin, can be spectrophotometrically titrated at pH 7.04. At pH 5.04, however, reaction between trypsin and NPABC is sufficiently slow for the reagent to be nearly specific for α -chymotrypsin. Specificity for one or other enzyme can be ensured by using soya-bean trypsin inhibitor or the chymotrypsin inhibitor L-1-chloro-3-toluene-*p*-sulphonamido-4-phenylbutan-2-one. Bovine thrombin does not react with NPABC. 4. Evidence is presented that indicates that acylation of α -chymotrypsin and trypsin by NPABC occurs at the active centres of the enzymes. 5. Evidence was obtained that indicates that one or more tryptophan residues move into a more hydrophobic environment when α -chymotrypsin and trypsin are acylated by NPABC.

The objectives and rationale of the present work were outlined in the introduction to the preceding paper (Elmore & Smyth, 1968). Although *N*-acetyl-L-phenylalanine ethyl ester is a good substrate for α -chymotrypsin, the nitrogen analogue ethyl N^2 -acetyl- N^1 -benzylcarbazate is not hydrolysed by the enzyme (Kurtz & Niemann, 1961*a*). It does inhibit chymotrypsin, however, probably not quite competitively, but nearly so. We may conclude that the compound is bound at the active site but does not acylate the enzyme. This is understandable, since the ester carbonyl carbon atom will have a lower fractional positive charge than a conventional substrate as a result of resonance with the adjacent nitrogen atom. It occurred to us that an aryl ester might be sufficiently reactive to acylate the enzyme, but that the acyl-enzyme would be relatively stable because, once again, the carbonyl carbon atom would have a relatively small fractional positive charge. These predictions were confirmed; NPABC* acylates α -chymotrypsin

stoichiometrically with the liberation of 1 mole of *p*-nitrophenol/mole of enzyme. Spectrophotometric determination of the amount of *p*-nitrophenol liberated leads to a rapid and simple assay of the amount of active enzyme in solution. Trypsin also reacts with the carbazate ester, but more slowly than α -chymotrypsin. This is a further illustration of the well-known cross-specificity of the two enzymes.

A preliminary account of some of this work has appeared (Elmore & Smyth, 1967).

EXPERIMENTAL

Materials

Trypsin, chymotrypsin and thrombin were the same samples used in the preceding paper (Elmore & Smyth, 1968). *N*-Methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine was prepared by the method of Fischer & Lipschitz (1915). N^2 -Acetyl- N^1 -benzylidenhydrazine was synthesized by the method of Kurtz & Niemann (1961*b*).

N^2 -Acetyl- N^1 -benzylhydrazine. A solution of N^2 -acetyl- N^1 -benzylidenhydrazine (2.0 g.) in dry tetrahydrofuran (130 ml.) was saturated with diborane at 0° and left at room temperature for 3 hr. A mixture of ethanol (50 ml.)

* Abbreviations: NPABC, *p*-nitrophenyl N^2 -acetyl- N^1 -benzylcarbazate; MTLNE, α -*N*-methyl- α -*N*-toluene-*p*-sulphonyl-L-lysine β -naphthyl ester.

and conc. HCl (2ml.) was added and solvent was removed under reduced pressure. The residue was taken up in water (20ml.) and extracted with ethyl acetate (5ml.). The aqueous solution was adjusted to pH 5.0 and extracted with four 30ml. portions of ether. The combined ethereal extracts were dried (MgSO₄) and concentrated under reduced pressure; crystallization was induced by the addition of light petroleum (b.p. 40–60°). The product (1.2g.) had m.p. 81–82°, unchanged on recrystallization from methylene dichloride–light petroleum (b.p. 60–80°) and agreeing with the value recorded in the literature (Kurtz & Niemann, 1961b). Reduction of *N*²-acetyl-*N*¹-benzylidenehydrazine with diborane is a convenient alternative to catalytic hydrogenation, which is attended by the risk of hydrogenolysis (Kurtz & Niemann, 1961b). Attempted acetylation of benzylhydrazine with thioacetic acid, acetyl chloride, acetic anhydride or *p*-nitrophenyl acetate gave intractable oils.

p-Nitrophenyl *N*²-acetyl-*N*¹-benzylcarbazate. A cold solution of *p*-nitrophenyl chloroformate (2.0g.) in dry chloroform (10ml.) was added during 30min. to a solution of *N*²-acetyl-*N*¹-benzylhydrazine (1.6g.) in dry chloroform (7.5ml.) containing triethylamine (1.02g.) at –5°; the triethylamine was distilled over sodium before use. The mixture was left overnight at room temperature and then washed with dil. HCl, water, ice-cold NaHCO₃ soln. and water and then dried (MgSO₄). Chloroform was removed under reduced pressure and the oily residue solidified on trituration with a few drops of ether. The product (2.3g.) had m.p. 116° after recrystallization from methylene dichloride–light petroleum (b.p. 60–80°) (Found: C, 58.5; H, 4.7; N, 12.6. C₁₆H₁₅N₃O₅ requires C, 58.4; H, 4.6; N, 12.8%).

N-Toluene-*p*-sulphonyl-L-phenylalanine β-naphthyl ester. This ester was synthesized by the interaction of di-β-naphthyl sulphite and *N*-toluene-*p*-sulphonyl-L-phenylalanine in dry pyridine in a manner similar to that described for the corresponding derivative of ε-*N*-benzyloxycarbonyl-α-*N*-toluene-*p*-sulphonyl-L-lysine (Elmore, Roberts & Smyth, 1967). After recrystallization from methylene dichloride–light petroleum (b.p. 60–80°), the ester (70%) had m.p. 136°, [α]_D²⁵ –7.0 ± 0.2° (c 5 in acetonitrile) (Found: C, 69.8; H, 5.3; N, 3.4. C₂₆H₂₃NO₄S requires C, 70.1; H, 5.2; N, 3.1%).

N-Methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine β-naphthyl ester. Similarly prepared from *N*-methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine, this ester (70%), after recrystallization from methylene dichloride–light petroleum, had m.p. 116°, [α]_D²⁵ +12.5 ± 0.3° (c 5 in acetonitrile) (Found: C, 70.5; H, 5.6; N, 3.4. C₂₇H₂₅NO₄S requires C, 70.6; H, 5.5; N, 3.0%).

N-Toluene-*p*-sulphonyl-L-phenylalanine 3-pyridyl ester hydrochloride. Esterification of *N*-toluene-*p*-sulphonyl-L-phenylalanine (10m-moles) by 3-hydroxypyridine (10m-moles) was accomplished in tetrahydrofuran (20ml.) in the presence of *NN'*-dicyclohexylcarbodi-imide (10m-moles). After filtration and concentration under reduced pressure, the oily residue solidified on the addition of ether containing HCl. The product (75%) crystallized from propan-2-ol-ether as a hemihydrate, having m.p. 173°, [α]_D²⁵ –37.5 ± 0.3° [c 5 in 30% (v/v) ethanol] (Found: C, 57.4; H, 4.7; N, 6.2. C₂₁H₂₁ClN₂O₄S·½H₂O requires C, 57.1; H, 5.0; N, 6.3%).

N-Methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine

3-pyridyl ester hydrochloride. Similarly prepared from *N*-methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine, this ester hydrochloride (75%) also crystallized as a hemihydrate, having m.p. 93°, [α]_D²⁵ –10.0 ± 0.2° (c 5 in acetonitrile) (Found: C, 58.3; H, 5.4; N, 6.6. C₂₂H₂₃ClN₂O₄S·½H₂O requires C, 58.0; H, 5.3; N, 6.1%). After the compound had been dried for 5hr. at 80° *in vacuo*, the m.p. changed to 118° and the analytical figures approximated to those required for the anhydrous compound.

N-Toluene-*p*-sulphonyl-L-valine 3-pyridyl ester. Prepared in a similar manner to the foregoing two compounds, this ester (75%) was obtained as the free base by recrystallization from methylene dichloride–light petroleum (b.p. 60–80°), and then had m.p. 153°, [α]_D²⁵ –39.5 ± 0.3° (c 5 in acetonitrile) (Found: C, 59.0; H, 5.6; N, 8.3. C₁₇H₂₀N₂O₄S requires C, 58.6; H, 5.8; N, 8.0%).

4-Carboxy-2-nitrophenyl 3-phenylpropionate. To a solution of 4-hydroxy-3-nitrobenzoic acid (10m-moles) in dry pyridine (20ml.), 3-phenylpropionyl chloride (10m-moles) was added slowly with stirring during 10min. The mixture was stirred for a further 20min. and filtered. The precipitated pyridinium salt was dissolved in boiling ethanol and an equivalent amount of dil. HCl was added. Water was added to precipitate the oily product, which then solidified on trituration. The product (70%) was recrystallized from methylene dichloride–light petroleum (b.p. 60–80°), when it had m.p. 161° (Found: C, 61.2; H, 4.5; N, 4.6. C₁₆H₁₃NO₆ requires C, 61.0; H, 4.2; N, 4.4%).

Methods

Determination of the absolute molarity of solutions of trypsin and chymotrypsin. An Optica CF4 single-beam spectrophotometer with ¼sec.-response Honeywell recorder was used as described in the preceding paper (Elmore & Smyth, 1968) except that an expanded scale was not required and matched 1cm. quartz cuvettes were used. A mixture of 0.05M-sodium cacodylate–HCl buffer, pH 5.04 or pH 7.04 (1ml.), mM-HCl (1ml.), 0.4mM-NPABC in 10% (v/v) propan-2-ol (1ml.) (freshly prepared by dilution of a solution of 4mM-NPABC in propan-2-ol) was placed in the reference cuvette and left to reach constant temperature. The sample cuvette initially contained buffer and substrate solutions (1ml. of each). The spectrophotometer was balanced through the reference cuvette. Enzyme in mM-HCl (1ml.) was added to the sample cuvette, the solution was mixed and recording at 340mμ (pH 5.04) or 400mμ (pH 7.04) was begun. From the height of the 'burst', the amount of *p*-nitrophenol liberated and hence the molarity of enzyme solution could be calculated. (For *p*-nitrophenol at pH 5.04, ε₃₄₀ is 6720; at pH 7.04, ε₄₀₀ is 8083.) Solutions of (*N*²-acetyl-*N*¹-benzyl)carbazoyl-trypsin and -chymotrypsin were prepared in a similar fashion at pH 7.04 for proflavine-binding and difference-spectral studies. After acylation was complete, the solutions of acyl-enzymes were dialysed in Visking tubing against running 0.2M-sodium acetate–acetic acid buffer, pH 5.0, for several hours. For titration with 2-hydroxy-5-nitrobenzyl bromide, acyl-enzyme solutions were dialysed against running mM-HCl.

Titration with 2-hydroxy-5-nitrobenzyl bromide. 2-Hydroxy-5-nitrobenzyl bromide (10mg.) (Koshland, Karkhanis & Latham, 1964) was added to a 40μM solution of enzyme or acyl-enzyme (10ml.) in dil. HCl at pH 3.0. The mixture was stirred at constant pH (pH-stat) for 30min. Excess of reagent was removed by gel filtration

on Sephadex G-25 in mM-HCl. The extinction was measured at $290\text{m}\mu$ and again at $410\text{m}\mu$ after the pH had been raised to 10.0 by addition of NaOH. Assuming that the modified tryptophyl residues had ϵ_{410} 18000 (Horton & Koshland, 1965), the number of such groups/mol. of protein could be calculated.

RESULTS

Reaction between NPABC and α -chymotrypsin.

The progress of the reaction between NPABC and α -chymotrypsin is shown in Fig. 1. Reaction was so fast at pH 7.04 that it was complete by the time that mixing was achieved and recording begun (less than 15 sec.). At pH 5.04 the reaction was complete in about 2 min. In both cases there was no further change in light-absorption within the time-scale of the experiment. Consequently, deacylation was negligible under these conditions. The molarity of a solution of α -chymotrypsin was determined at pH 7.04 and pH 5.04 with NPABC and also by the method of Erlanger & Edel (1964) with 4-carboxy-2-nitrophenyl *NN*-diphenylcarbamate. The values obtained were respectively $53.1\text{ }\mu\text{M}$, $55.2\text{ }\mu\text{M}$ and $53.5\text{ }\mu\text{M}$. In another experiment it was found that the amount of *p*-nitrophenol liberated by a fixed amount of α -chymotrypsin was independent of the concentration of NPABC, provided that this exceeded the enzyme concentration. Moreover, by using accurate dilutions of a stock solution of α -chymotrypsin, it was found that the amount of *p*-nitrophenol liberated was proportional to the amount of enzyme present.

There was no liberation of *p*-nitrophenol when NPABC was added to a solution of α -chymotrypsin that had previously been inhibited with L-1-chloro-3-toluene-*p*-sulphonamido-4-phenylbutan-2-one. Conversely, α -chymotrypsin that had been acylated with NPABC did not bind proflavine at pH 5.0.

Other compounds were examined as possible alternative reagents for determining the absolute molarity of chymotrypsin solutions. *N*-Toluene-*p*-sulphonyl-L-phenylalanine β -naphthyl ester and *N*-methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine β -naphthyl ester were too insoluble in aqueous solvents. *N*-Toluene-*p*-sulphonyl-L-phenylalanine 3-pyridyl ester, *N*-methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine 3-pyridyl ester, *N*-toluene-*p*-sulphonyl-L-valine 3-pyridyl ester and 4-carboxy-2-nitrophenyl 3-phenylpropionate were all hydrolysed by α -chymotrypsin, but in each case the turnover rate was too great relative to the rate of acylation for them to be of practical use in the titration of the enzyme.

Reaction between NPABC and trypsin. The progress of the reaction between NPABC and trypsin is also shown in Fig. 1. Trypsin was acylated much more slowly than α -chymotrypsin by

Fig. 1. Time-course of the acylation of trypsin and α -chymotrypsin by NPABC: reaction with α -chymotrypsin ($54\text{ }\mu\text{M}$) at pH 7.04 (I) and pH 5.04 (III); reaction with trypsin ($44\text{ }\mu\text{M}$) at pH 7.04 (II) and pH 5.04 (IV). Other experimental details are given in the Experimental section.

NPABC; the rate of acylation of trypsin at pH 7.04 was approximately equal to the rate of acylation of α -chymotrypsin at pH 5.04. At pH 5.04, trypsin reacted very slowly with NPABC. We assayed a solution of trypsin with NPABC at pH 7.04 and with MTLNE (Elmore & Smyth, 1968) at pH 4.0 and obtained values of $43.9\text{ }\mu\text{M}$ and $45.0\text{ }\mu\text{M}$ respectively. In the presence of excess of soya-bean trypsin inhibitor, trypsin did not react with NPABC. Conversely, trypsin, like α -chymotrypsin, after it had been acylated with NPABC did not bind proflavine at pH 5.0, did not liberate *p*-nitrophenol from α -*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester at pH 3.0 and did not liberate β -naphthol from MTLNE at pH 4.0.

Thrombin did not liberate *p*-nitrophenol from NPABC at pH 7.04, nor did it catalyse the hydrolysis of *N*-methyl-*N*-toluene-*p*-sulphonyl-L-phenylalanine 3-pyridyl ester, *N*-toluene-*p*-sulphonyl-L-valine 3-pyridyl ester or 4-carboxy-2-nitrophenyl 3-phenylpropionate at pH 6.0. α -*N*-Toluene-*p*-sulphonyl-L-phenylalanine 3-pyridyl ester was hydrolysed by thrombin, but the ratio of the rates of acylation and deacylation was not high enough for the substrate to be of practical value for the assay of solutions of thrombin.

Some properties of (N²-acetyl-N¹-benzyl)carbazoyl-trypsin and -chymotrypsin. The difference spectra of the acyl-enzymes versus the native enzymes at pH 5.0 had peaks at 282m μ and 290m μ and resembled the difference spectra recorded by Wootton & Hess (1962) and Havsteen, Labouesse & Hess (1963) for di-isopropylphosphoryl- and acetyl-chymotrypsin versus the native enzymes. The peak at 290m μ was more pronounced for (N²-acetyl-N¹-benzyl)carbazoyl-chymotrypsin than for the corresponding trypsin derivative. The accessibility of the tryptophyl residues in the native enzymes and their acyl derivatives was assessed by titration with 2-hydroxy-5-nitrobenzyl bromide at pH 3.0. The numbers of accessible tryptophyl residues/mol. found were as follows: trypsin, 2.0; (N²-acetyl-N¹-benzyl)carbazoyl-trypsin, 1.2; α -chymotrypsin, 2.5 (cf. Koshland *et al.* 1964); (N²-acetyl-N¹-benzyl)carbazoyl-chymotrypsin, 0.3.

DISCUSSION

Trypsin and α -chymotrypsin readily react with NPABC and each liberates 1 mole of *p*-nitrophenol/mole of active enzyme. Parallel assays by the methods of Erlanger & Edel (1964) and Elmore & Smyth (1968) confirm the stoichiometry of the reactions. Although the rates of liberation of *p*-nitrophenol are strongly pH-dependent as expected, the total amount of *p*-nitrophenol is not. Bender *et al.* (1966) have already shown that titrations of the active site of α -chymotrypsin are independent of pH over a wide range.

The rapidity of formation and stability of the acyl-enzymes formed by the interaction of NPABC with trypsin and α -chymotrypsin offer an important advantage over most of the earlier methods for the titration of trypsin and chymotrypsin. Self-digestion of the enzymes during acylation is negligible and runs do not have to be carried out at different concentrations of titrant. Moreover, bovine thrombin, which hydrolyses α -N-benzyl-oxycarbonyl-L-lysine *p*-nitrophenyl ester, is insensitive to NPABC. The ability of NPABC to react with both trypsin and α -chymotrypsin, albeit at widely different rates, provides a further example of the cross-specificity of these enzymes (Inagami & Sturtevant, 1960). This lack of discrimination between trypsin and chymotrypsin can easily be overcome, however, and the individual enzymes can be assayed in a mixture. The reaction between NPABC and trypsin is much slower than the corresponding reaction with α -chymotrypsin (Fig. 1), so that the latter enzyme can be assayed directly at about pH 5 with only a small error due to *p*-nitrophenol liberated by trypsin. For example, an assay of a 1:1 mixture of α -chymotrypsin and trypsin would only have an error of about 5% due to con-

taminating trypsin provided that the extinction was determined after 2–3 min. at 25°. Alternatively, the activities of trypsin and α -chymotrypsin towards NPABC can be completely and selectively inhibited by pretreatment with soya-bean trypsin inhibitor and L-1-chloro-3-toluene-*p*-sulphonamido-4-phenylbutan-2-one respectively.

It is important to establish that NPABC and MTLNE react only at the active centres of α -chymotrypsin and trypsin. A considerable body of evidence supports this view. (1) Kurtz & Niemann (1961a) showed that the ethyl ester analogous to NPABC inhibits α -chymotrypsin almost competitively and is presumably bound at the active site. (2) Proflavine is bound by α -chymotrypsin in the pH range 4.0–7.6, but not if the enzyme is pretreated with L-1-chloro-3-toluene-*p*-sulphonamido-4-phenylbutan-2-one, phenyl-methanesulphonyl fluoride or *N*-cinnamoylimidazole (Glazer, 1965; Bernhard, Lee & Tashjian, 1966). The first of these compounds reacts with His-57 whereas the last two react with Ser-195. Since proflavine does not bind to α -chymotrypsin that has been treated with NPABC, it may be concluded that the latter blocks the active centre of the enzyme. (3) Proflavine is also bound by trypsin, but not in the presence of soya-bean trypsin inhibitor or α -N-benzoyl-L-arginine ethyl ester (Glazer, 1965; Bernhard & Gutfreund, 1965). Since proflavine does not bind to trypsin that has been acylated with NPABC, evidently the latter reacts at the active centre of trypsin. (4) After treatment with L-1-chloro-3-toluene-*p*-sulphonamido-4-phenylbutan-2-one, chymotrypsin no longer liberates *p*-nitrophenol from NPABC. (5) Trypsin does not react with NPABC in presence of soya-bean trypsin inhibitor. (6) Trypsin that has been allowed to react with NPABC does not liberate *p*-nitrophenol from α -N-benzoyloxycarbonyl-L-lysine *p*-nitrophenyl ester or β -naphthol from MTLNE.

Wootton & Hess (1962) and Havsteen *et al.* (1963) have shown that there are small but significant differences between the spectra of α -chymotrypsin on the one hand and its di-isopropylphosphoryl and acetyl derivatives on the other. They concluded that a tryptophyl residue became less accessible to water when the enzyme was acylated at Ser-195. The difference spectra of the N²-acetyl-N¹-benzylcarbazoyl derivatives of chymotrypsin and trypsin versus the free enzymes resemble the spectra recorded by Wootton & Hess (1962) for acyl-chymotrypsins and it appears that a similar explanation is applicable to our derivatives. Support for this view was obtained by titration of accessible tryptophyl residues in the free enzymes and their acyl derivatives with 2-hydroxy-5-nitrobenzyl bromide. The number of accessible

tryptophyl residues/mol. falls by about 1 in trypsin and by about 2 in α -chymotrypsin after acylation with NPABC.

One facet of our work is the failure to find a suitable reagent for the titration of bovine thrombin. The latter appears to have a much narrower specificity than either chymotrypsin or trypsin, in spite of its ability to hydrolyse *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester, *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester and methyl esters of L-phenylalanine, L-tyrosine and L-tryptophan (Lorand, Brannen & Rule, 1962; Cole, Koppel & Olwin, 1967), in addition to some of the typical synthetic substrates of trypsin. Moreover, with a few exceptions, such as the hydrolysis of α -*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (Kézdy, Lorand & Miller, 1965) and esters of α -*N*-benzoyl-L-arginine (J. B. Baird & D. T. Elmore, unpublished work), there is a lack of evidence that thrombin-catalysed reactions proceed by the acylation-deacylation mechanism.

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