Studies on Ram Acrosin

FLUORIMETRIC TITRATION OF OPERATIONAL MOLARITY WITH 4-METHYLUMBELLIFERYL *p*-GUANIDINO BENZOATE

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1. Titration in sodium barbiturate buffer of acrosin, a serine proteinase from sperm acrosomes, with the ester substrate 4-methylumbelliferyl *p*-guanidinobenzoate gave rise to an incomplete 'burst' of 4-methylumbelliferone. Studies of the effects on the reaction of activators of acrosin (Ca^{2+} , water-miscible solvents) showed that titrations carried out in barbiturate buffer containing 1 M- CaCl_2 and diluted with 0.2 vol. of dimethyl sulphoxide produced a rapid quantitative burst within 4 min at 20°C. 2. The net post-burst production of 4-methylumbelliferone was negligible because (a) the acyl-enzyme was very stable, and (b) the slow post-burst formation of 4-methylumbelliferone (turnover of acyl-enzyme) was virtually equal to the slow photolytic destruction of 4-methylumbelliferone that was liberated during the burst. 3. The standard procedure permits titrations of 20–100 pmol of acrosin, i.e. amounts normally taken for conventional rate assays, and with these amounts the impurities present in crude enzyme fractions did not interfere. The burst was judged to be quantitative on the basis of comparisons with titrations of acrosin with *p*-nitrophenyl *p'*-guanidinobenzoate. 4. The burst reaction of trypsin with the 4-methylumbelliferyl ester was inhibited by high Ca^{2+} concentrations and by dimethyl sulphoxide. 5. The association and dissociation of complexes of both acrosin and trypsin with protein-type inhibitors (Kunitz pancreatic trypsin inhibitor and a spermatozoal acrosin inhibitor) are rather slow. It is thus possible, in certain cases, to use the ester to titrate both total enzyme in an inhibitor–enzyme mixture and net enzyme, i.e. the stoichiometric excess of enzyme over inhibitor.

Jameson *et al.* (1973) have described active-site titrations of the serine proteinases α - and β -trypsins, thrombin and factor Xa with the ester substrate 4-methylumbelliferyl *p*-guanidinobenzoate (I). Acyl-enzyme intermediates were sufficiently stable to allow initial 'bursts' of 4-methylumbelliferone (II) to serve as measures of the operational molarities of the enzyme solutions. The advantage of this procedure is that, with fluorimetric determination of compound II, the titration can be at least 100 times more sensitive than customary spectrophotometric methods, e.g., with *p*-nitrophenyl *p'*-guanidinobenzoate (Chase & Shaw, 1970). Active-site titrations have hitherto been carried out mainly with extensively purified enzymes (Bender *et al.*, 1966; Jameson *et al.*, 1973; also references quoted in the latter paper). This is often obligatory for spectrophotometric methods, where accuracy calls

for active-site concentrations of 5–10 μM . Extraneous protein (and other contaminants) of non-purified enzymes can interfere with the spectrophotometric assay of *p*-nitrophenol, e.g. through co-precipitation. The much lower concentration of proteinase required for fluorimetric assay decreases the risk of interference in addition to conserving stocks of scarce materials. It therefore seemed to us that direct fluorimetric measurements of enzyme concentration could well replace customary assays, based on rates of substrate hydrolysis, at most if not all stages of purification of a serine proteinase.

We made the assumption that accurate active-site titrations are most likely to be achieved in conditions favouring a rapid reaction of proteinase with titrant. Making use of our experience with activators of acrosin (Brown *et al.*, 1975) we have developed a fluorimetric procedure for assaying the molarity of ram acrosin solutions with compound I, a procedure that is applicable to crude enzyme fractions. The experiments in this paper support the view that the mechanism of the acrosin–compound I reaction

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may be represented in the same way as the general scheme for trypsin:

where ES' is the acyl-enzyme and P₁ is compound II.

Materials

Sucrose, analytical-grade, was from Fison's Scientific Ltd., Loughborough, Leics., U.K.; compound II was an Eastman product, m.p. 188–189°C, supplied by Kodak Ltd., Kirkby, Liverpool, U.K. Crystalline bovine trypsin, Trasylol (Kunitz bovine basic pancreatic trypsin inhibitor), acrosomal inhibitor (an inhibitor of acrosin and trypsin that was isolated from ram spermatozoa) and dimethyl sulphoxide were as described in Brown *et al.* (1975). *p*-Nitrophenyl *p*'-guanidinobenzoate hydrochloride was a gift from Dr. E. Shaw (Brookhaven National Laboratories, Upton, N.Y., U.S.A.).

The hydrochloride of compound I was prepared by condensing *p*-guanidinobenzoic acid hydrochloride (Chase & Shaw, 1970) with compound II as described by Jameson *et al.* (1973), but with changes in the purification procedure. It was crystallized twice from acetic acid (crop 1, yield 65%). The product was not obviously crystalline. Attempts to crystallize crop 1 from 2-methylbutan-2-ol–diethyl ether (Jameson *et al.*, 1973) were unsuccessful. Part of crop 1 was dissolved in hot cyclohexanone, diluted with an equal volume of hot ethanol, and filtered. The solution was stored at room temperature as a 2–3 cm layer in an open flask for 4 days. White needles (crop 2) were deposited. Both crops were dried to constant weight *in vacuo* over P₂O₅. Crop 1 had m.p. 180–185°C (Found: C, 56.35; H, 4.50; N, 11.02; Cl, 9.16. C₁₈H₁₆N₃ClO₄·½H₂O requires C, 56.47; H, 4.32; N, 10.98; Cl, 9.27%). Crop 2 had m.p. 209–212°C (Found: C, 58.00; H, 4.37; N, 11.36; Cl, 9.32. C₁₈H₁₆N₃ClO₄ requires C, 57.83; H, 4.32; N, 11.24; Cl, 9.48%). Jameson *et al.* (1973) give m.p. 219–221°C for a monohydrate.

Ram acrosin

Three preparations were used: fraction S1/2, a crude acid extract (pH 2.7) of 'denuded' spermatozoa; fraction FD, a freeze-dried product representing a partial purification of fraction S1/2; and an inhibitor-free freeze-dried product obtained from fraction FD by chromatography on a column of Sephadex G-75. For details see Brown *et al.* (1975). The freeze-dried enzyme preparations were used as 0.2% solutions in 1 mM-HCl. Unless otherwise stated experiments were carried out with the inhibitor-free preparation.

Buffers

Compound I, like *p*-nitrophenyl *p*'-guanidinobenzoate, is more stable in barbiturate buffers than in Tris buffers. Titrations of acrosin (and trypsin) with compound I were followed in 0.1M-sodium barbiturate-HCl buffers, pH 8.2, containing 0–1.43M-CaCl₂. In work with acrosin these buffers were usually diluted with 0.2 vol. of dimethyl sulphoxide. If CaCl₂ exceeds 0.2M the addition of the solvent is obligatory, otherwise barbiturate crystallizes. Buffers containing dimethyl sulphoxide were stored at 5°C: after several weeks on the laboratory bench they become inhibitory towards the reaction of acrosin with compound I. The term 'standard buffer' will be used for 0.1M-barbiturate–1.0M-CaCl₂, pH 8.2, diluted with 0.2 vol. of dimethyl sulphoxide.

Titration reagents

Standard (5 mM) solutions of compounds I and II were prepared in *N*-methylpyrrolid-2-one (Jameson *et al.*, 1973). They were stable for 6 months at –20°C. Appropriate dilutions were made daily in 1 mM-HCl and stored below 20°C. Hydrolysis of compound I occurs at a significant rate at higher temperatures.

Methods

Determinations of operational molarities of acrosin (and trypsin)

The method of Chase & Shaw (1970) for titrating trypsin with *p*-nitrophenyl *p*'-guanidinobenzoate was used by Schiessler *et al.* (1972) for titrating boar acrosin. We have used the same method for ram acrosin except that 0.2M-CaCl₂ was present and the substrate solution was made up as described by Jameson *et al.* (1973).

Fluorescence of compound II was measured at 20±0.5°C in an Aminco-Bowman ratio-recording spectrophotofluorimeter with wavelength settings 363 nm (excitation) and 457 nm (emission). Entrance and exit slits were 2 and 4 mm respectively.

Fluorimetric procedure. To assay 10–100 pmol of acrosin (or trypsin) photometric sensitivity is adjusted so that fluorescence of 50 nM-compound II [in sodium barbiturate buffer–dimethyl sulphoxide (5:1, v/v)] in a standard cuvette (10 mm×10 mm) gives rise to about 40% of full-scale deflexion on an external recorder set at 20 mV. A cuvette containing 1 ml of buffer is placed in the fluorimeter and the pen is brought to the left side of the recorder chart which is running at 1 cm/min. (Appropriate buffers are described in the Results section.) The cuvette next receives 5 μl of 0.1 mM-compound I (500 pmol): this should give rise to a deflexion of not more than 2% of full scale. The enzyme solution (up to 10 μl) is then added. The initial burst of compound II and any subsequent slow production of compound II

are followed for 4–10 min. After 10 min, or earlier if the pen becomes stationary, the pen is backed off (if necessary) to the left half of the chart and, 10 s later, 5 μ l of 0.01 M-compound II is added to obtain a standard deflexion for 50 pmol of compound II. The sensitivity of the assay can be increased at least fivefold, without increase in instrumental 'noise', by the use of Aminco-Bowman micro-cuvettes (diameter 3 mm). The two crops of compound I gave identical results.

For certain types of experiment it was necessary to initiate the burst by added compound I to buffered acrosin. These were: (a) experiments on the effects of inhibitors, where a prior 5 min contact of enzyme and inhibitor at pH about 8 was necessary; (b) titrations of acrosin fraction S1/2, since such fractions developed fluorescence (equivalent to 2–4 pmol of compound II) when added to barbiturate buffers.

Results

Exploratory experiments: influence of Ca²⁺, organic solvents and sucrose on the reaction of acrosin with compound I

Except where otherwise stated the experiments in this section were carried out with a single preparation of acrosin fraction FD.

Addition of 10 μ g of acrosin to 1 ml of 0.1 M-sodium barbiturate buffer containing 500 pmol of compound I gave rise to an immediate 'burst' of compound II, followed by slow production of compound II, presumably owing to turnover of the acyl-enzyme. The turnover phase was not linear but progressed at a decreasing rate for 20 min: thus extrapolation to zero time was impossible. The velocity of the burst reaction increased considerably in the presence of CaCl₂ at concentrations up to 0.2 M. With Ca²⁺ present the turnover phase was not linear at first, but after 8 min the rate of formation of compound II was very low and virtually constant. Several water-soluble organic solvents activate trypsin (Castañeda-Agulló & del Castillo, 1959) and the same solvents also activate acrosin in rate assays with synthetic substrates (Brown *et al.*, 1975). Inclusion of 2-methylpropan-2-ol or dimethyl sulphoxide (20%, v/v) in the barbiturate buffer increased the burst velocity of the reaction and after 6 min the turnover phase was slow and linear.

Acrosin fraction S1/2, a low-pH extract of spermatozoa, becomes cloudy at pH 8 and the enzyme precipitates (Brown *et al.*, 1975). Attempts to titrate fractions S1/2 with compound I in barbiturate buffers containing 0.2 M-CaCl₂, 2-methylpropan-2-ol, or both were unsuccessful owing to progressive increase in opalescence. However, the presence of both dimethyl sulphoxide and CaCl₂ (Table 1) prevented loss of transparency and permitted titrations of S1/2 fractions. An incidental advantage of

Table 1. *Fluorescence at 20°C of 50 nM-compound II in buffers based on 0.1 M-sodium barbiturate, pH 8.2*

See under 'Exploratory experiments' in the Results section.

Additions to buffer	Fluorescence relative to (1)
(1) 0.2 M-CaCl ₂	[100]
(2) 0.2 vol. of Me ₂ SO	111
(3) 0.2 M-CaCl ₂ -0.1 M-sucrose	110
(4) 0.2 M-CaCl ₂ -0.2 vol. of Me ₂ SO	124

Table 2. *Release of compound II from compound I by an acrosin fraction FD: effect of adding dimethyl sulphoxide to the buffer medium (0.1 M-barbiturate-0.2 M-CaCl₂)*

Reactions were carried out in 1.0 ml of buffer or of buffer-dimethyl sulphoxide. The burst was determined by extrapolation to zero time of the linear rate after 8 min (see the text).

Volume of Me ₂ SO added to 1 vol. of buffer	Burst produced by 10 μ g of acrosin (pmol)
0*	45.1
0.04	48.5
0.08	47.8
0.15	48.7†
0.20*	49.4†
0.25	43.2
0.30	40.0

* Buffers 1 and 4 of Table 1.

† Increase in concentration of compound II ceases after 4 min.

this solvent is that it enhances the fluorescence of compound II.

Further experiments (Table 2) revealed an optimum value (in terms of the magnitude of the burst) for the ratio dimethyl sulphoxide/buffer. Further, when the ratio was 0.15–0.20 the post-burst formation of compound II was negligible. The essential points from these experiments are illustrated in Fig. 1. Buffer 4 gave rise to a higher burst than did the others if extrapolation was carried out. We accept buffer 4 values for the burst as being the most accurate since they agreed within 5% with titrations performed with *p*-nitrophenyl *p*'-guanidinobenzoate. To compare the two methods we used 0.8–1.6 mg of acrosin with the *p*-nitrophenyl ester and 5–10 μ g of acrosin with compound I.

Since we had planned to study the effects of Trasylol on the reaction of compound I with acrosin, and since solutions of Trasylol in 0.25 M-sucrose had been prepared for other experiments, we studied the effect of sucrose on the titration with compound I. As Fig. 1 shows, sucrose causes a considerable

Fig. 1. Fluorimetric titration of acrosin fraction FD with compound I: time-course of release of compound II after addition of 10 μ g of acrosin to 500 pmol of compound I in 1 ml of buffer

Curves are numbered to correspond with buffers in Table 1.

Fig. 2. Production of compound II from compound I in buffers containing 0.2M-CaCl₂ and sucrose or glycerol

●, Post-burst rates, as a function of sucrose concentration, in experiments as Fig. 1, Expt. 3; ○, rates in controls without enzyme; ■, post-burst rate in the presence of glycerol.

increase in the post-burst hydrolysis of compound I. Incorporation of sucrose at concentrations up to 0.4M gave rise to still greater post-burst rates (Fig. 2). Controls without enzyme also exhibited hydrolysis

rates that increased with sucrose concentration. Thus rates shown by the curve of Fig. 2 arise mainly from an acrosin-independent, but sucrose-dependent, hydrolysis. Glycerol had a significant but much smaller catalytic power. The sucrose effect appears to be intrinsic and not due to contaminating esterase activity. Buffers (0.1M-barbiturate-0.2M-CaCl₂) were made up containing 0.4M-sucrose from various sources [cane sugar (Tate and Lyle Ltd., Mincing Lane, London E.C.3, U.K.), beet sugar (British Sugar Corp., Tower Hill, London E.C.3, U.K.) and a highly purified sucrose (Aristar, BDH Chemicals, Poole, Dorset, U.K.)]. In addition, the different sucroses were autoclaved, as 1M-solutions, for 2h at 120°C and incorporated at 0.4M in barbiturate-CaCl₂ buffer. The rates of hydrolysis of 500 pmol of compound I in 1 ml of each of these buffers agreed within 6% of the mean value (4.0 pmol/min).

The negligible post-burst turnover in barbiturate-0.2M-CaCl₂-dimethyl sulphoxide buffer suggested that it might be the ideal medium for titration of acrosin. However, later experiments showed that with all samples of inhibitor-free acrosin, and even with some samples of acrosin fraction FD, a quite high post-burst turnover occurred in this buffer.

Influence of Ca²⁺ concentration on the reactions of acrosin and trypsin with compound I

Acrosin. Fig. 3 shows the effects of up to 1.43M-CaCl₂ on the titration of acrosin in buffers containing dimethyl sulphoxide. Increasing CaCl₂ concentrations suppressed the turnover, but concentrations above 1.2M inhibited the burst rate. All three types of ram acrosin were tested in standard buffer (i.e. with 1M-CaCl₂ present) and if the quantities of

Fig. 3. Titration curves of 10 μ g of acrosin in buffer-dimethyl sulphoxide (5:1, v/v)

The buffer (0.1M-barbiturate, pH 8.2) contained the following molarities of CaCl₂: (a) 0.20; (b) 0.57; (c) 0.75; (d) 0.98; (e) 1.20; (f) 1.43.

compound I and enzyme were 500 and 25–75 pmol respectively the post-burst turnover was insignificant. Replacement of CaCl_2 by MgCl_2 led to essentially identical results. Comparisons of titrations of acrosin (a) with compound I in standard buffer, and (b) with *p*-nitrophenyl *p*'-guanidinobenzoate in 0.1 M-barbiturate–0.2 M- CaCl_2 again gave good agreement, i.e. within 5%.

Trypsin. Titrations in the buffers listed in Fig. 3 showed that the burst rate was progressively inhibited by CaCl_2 concentrations above 0.2 M. Jameson *et al.* (1973) used a calcium-free barbiturate buffer for titrating trypsin. We found that addition of up to 0.2 M- CaCl_2 made the burst more rapid and slightly decreased the post-burst turnover rate. We therefore use buffer 1 (Table 1) for routine assays of trypsin. Addition of dimethyl sulphoxide to such buffers inhibits the burst rate considerably.

Optimum conditions for fluorimetric assay of acrosin: relationship between burst and concentration of enzyme

Fig. 4 shows results from titrations, in 1 ml of standard buffer containing 500 pmol of compound I, of 2–20 μg of acrosin. The experimental points (filled circles) are based on pen deflexions at 4 min after addition of enzyme. With enzyme/substrate ratios ($[\text{E}]_0/[\text{S}]_0$) between 0.05 and 0.15, the pen became stationary after 3–3.5 min and π , the magnitude of the

Fig. 4. Relationship of the burst to weight of acrosin

Assays were as in Fig. 3 but with standard buffer. ●, Burst determined from recorder-pen deflexion 4 min after adding acrosin; ○, burst calculated from extrapolation back to time of addition of acrosin (where this method gives a different burst value).

Table 3. Side reactions of compound I accompanying the formation of acyl-acrosin

Cuvettes received 1 ml of standard buffer followed by additions shown below. (A) Controls without enzyme. (B) Titrations of acrosin at two temperatures; also controls without enzyme. Rates in (B) were measured with intermittent irradiation and with a 2.5-fold increase in sensitivity (possible error: ± 0.04).

Temperature (°C)	Compound I (pmol)	Acrosin (pmol)	Rate of production of compound II	
			Before adding 50 pmol of compound II (pmol/min)	After adding 50 pmol of compound II (pmol/min)
(A) 20	500	—	0.4	–0.2
20	1000	—	1.5	0.6
20	2000	—	4.1	2.8
20	4000	—	8.4	6.9
20	—	—	—	–0.4
(B) 20	500	52	0.39*	—
25	500	52	0.81*	—
20	500	—	0.37	—
25	500	—	0.51	—

* Post-burst rates.

burst (Bender *et al.*, 1966), was linearly related to the weight of acrosin. When the ratio 0.15 was exceeded, π reached a maximum at about 3 min and then decreased very slowly. This arises from photolytic breakdown of compound II (see below). Experiments in which $[\text{E}]_0/[\text{S}]_0$ exceeded 0.2 showed progressively increasing divergences from linearity owing to incomplete formation of ES. When the ratio was less than 0.05 the burst was followed by a slow turnover. Extrapolation to zero time in such cases gave π values (open circles) which tended to lie closer to the linear relationship in Fig. 4. Provided that the ratio is of the order of 0.1 it is possible, by changing the concentration of compound I, to vary the sensitivity of the method to cover different acrosin ranges, e.g. 2.5–25, 25–250 pmol. Increasing the quantity of compound I above 1500 pmol leads to an inconveniently high rate of spontaneous hydrolysis (see below).

Side reactions accompanying and following the burst reaction

Photodecomposition of compound II during titrations of α -trypsin with compound I was noted by Jameson *et al.* (1973), who eliminated it by irradiating the cuvette intermittently. We have titrated acrosin in standard buffer by such a method (1.5 s irradiation at 0.5 min intervals) and have found that pre- and post-burst rates are almost identical. Further, back-extrapolation of the latter rates gave results that were not significantly different from

results obtained with continuous irradiation, which eliminated net post-burst changes in the concentration of compound II.

The recorded post-burst rates in our experiments, after correction for spontaneous hydrolysis, should equal the rate of turnover of the acyl-enzyme less the rate of photolysis of compound II. Spontaneous hydrolysis was not markedly affected by the presence of CaCl_2 or dimethyl sulphoxide. A study of this hydrolysis, as well as of post-burst changes (Table 3), revealed the following points.

(1) Rates of spontaneous hydrolysis of compound I increased with concentration (A). When a standard 50 pmol of compound II was subsequently added the rates fell and even became negative (in terms of concentration of compound II). That photolysis of compound II occurs is confirmed by its slow disappearance from the control without compound I. No loss of compound II occurred when the same control was run with intermittent irradiation.

(2) Comparison of pre- and post-burst rates in acrosin titrations at 20°C and 25°C (B) were carried out with intermittent irradiation. The spontaneous (pre-burst) hydrolysis had a large temperature coefficient and the post-burst rate had a still larger one. A noteworthy finding was that at 20°C the pre- and post-burst rates of hydrolysis of compound I did not differ significantly (0.37, 0.39), i.e. the acyl-enzyme is very stable in standard buffer at this temperature. Further, in 1 ml of standard buffer containing 450 pmol of compound I and 50 pmol of compound II (conditions prevailing immediately after the burst promoted by 50 pmol of acrosin) the rate of formation of compound II by spontaneous hydrolysis was almost cancelled out by the rate of photolysis of liberated compound II.

Effect of concentration of compound I on π at a constant acrosin concentration

On the basis of a kinetic analysis by Gutfreund & Sturtevant (1956) of chymotrypsin catalysis, Bender *et al.* (1966) derived the equation:

$$\pi = [E]_0 \left(\frac{k_2}{k_2 + k_3} \right)^2 \left/ \left(1 + \frac{K_m}{[S]_0} \right) \right. \quad (2)$$

A good enzyme titrant must satisfy the condition $k_2 \gg k_3$ and $[S]_0 \gg K_m$, so that π becomes equal to $[E]_0$. The progress of fluorimetric titration indicates that the former condition holds for the reaction of acrosin with compound I in standard buffer. Thus eqn. (2) may be simplified and rearranged to give eqn. (3)

$$\frac{1}{\sqrt{\pi}} = \frac{1}{\sqrt{[E]_0}} + \frac{K_m}{[S]_0 \sqrt{[E]_0}} \quad (3)$$

from which it follows (Bender *et al.*, 1966) that a plot of $1/\sqrt{\pi}$ against $1/[S]_0$ will intercept the ordinate at $1/\sqrt{[E]_0}$.

Data for such a plot were obtained by titrating 10 μg of acrosin with various amounts of compound I.

Fig. 5. Representative titrations in standard buffer of 10 μg of acrosin with different quantities of compound I

(a) 150 pmol; (b) 500 pmol; (c) 4000 pmol. The three π values, with others, are utilized in Fig. 6. ----, Spontaneous hydrolysis of compound I before addition of acrosin (arrow).

Fig. 6. Variation in π with concentration of compound I (plotted as described by Bender *et al.*, 1966)

Points (a), (b) and (c) are derived from Fig. 5. Point (b), representing standard conditions for titration, was used to calculate $[E]_0$.

Some of the recorder traces are illustrated in Fig. 5. With 150 pmol of compound I the burst was apparently incomplete within 10 min (curve a). With 300–500 pmol a normal type of titration curve was obtained (e.g. curve b). With 1000 pmol the pre- and post-burst rates were significant and extrapolation was necessary for determining the burst. With still higher quantities of compound I these rates were considerable, e.g. curve c (4000 pmol). The plot of $1/\sqrt{\pi}$ against $1/[S]_0$ (Fig. 6) reveals virtually no change in π over the middle section ($[E]_0/[S]_0 = 0.05$ – 0.15) where titration was complete within 4 min. When pre- and post-burst rates are high and the burst rate very rapid ($1/[S]_0 \gg 0.5$) manual addition of enzyme to the cuvette, and subsequent mixing, could not be

timed as accurately as the assay demands. This difficulty, together with the fact that post-burst rates are somewhat lower than pre-burst rates, adds uncertainty to π values. In such assays these values were consistently 5–10% greater than those obtained with lower concentrations of compound I. It may, however, be concluded that $[S]_0 \gg K_m$ in normal assay conditions and that extrapolation on the basis of Fig. 6 is unnecessary.

With provision of means for accurate timing of addition of acrosin, and simultaneous rapid mixing, Expt. c (Fig. 5) could become the basis of a very rapid fluorimetric titration.

Fluorimetric titration of acrosin and trypsin in the presence of inhibitors

Acrosin-acrosomal inhibitor. The two upper curves of Fig. 7 illustrate the influence of up to 30 μg of acrosomal inhibitor on the titration of 42 pmol of acrosin and the effect of briefly storing enzyme and inhibitor together, in standard buffer, before adding compound I. Curve *a*, taken from Brown *et al.* (1975), is a determination of the stoichiometric relationship of the acrosin and inhibitor preparations by extrapolation of the linear portion of the curve. Thus by the fluorimetric method acrosin may be assayed in the presence of a considerable molar excess of inhibitor. This could be of practical value in view of the widespread occurrence of the acrosomal inhibitor in spermatozoa.

Trypsin-Trasytol and acrosin-Trasytol. Trypsin associates stoichiometrically (1:1) with Trasytol, and the equilibrium constant is so low ($6 \times 10^{-14} \text{ M}$) that complete inhibition of the hydrolysis of *p*-tosyl-

Fig. 7. Fluorimetric titrations of acrosin in the presence of different amounts of acrosomal inhibitor

●, Burst was initiated by adding 8 μg of enzyme to standard buffer containing inhibitor and substrate (500 pmol of compound I). ○, Buffer+enzyme+inhibitor was stored for 5 min and the burst was initiated by adding substrate. (a) Curve from Fig. 9 of Brown *et al.* (1975): hydrolysis of *N*- α -benzoyl-DL-arginine 2-naphthylamide by mixtures of different amounts of inhibitor with 8 μg of the acrosin preparation used for the fluorimetric titrations.

Fig. 8. Fluorimetric titrations of trypsin and acrosin in the presence of Trasytol

Trypsin (73 pmol) was titrated in 0.1 M-barbiturate-0.2 M- CaCl_2 ; acrosin (59 pmol) was titrated in standard buffer. □, Trypsin, Trasytol and buffer were mixed and 500 pmol of compound I was added 5 min later; ■, trypsin was added to a mixture of buffer, Trasytol and compound I. ○, As □ but with acrosin; ●, as ■ but with acrosin.

L-arginine methyl ester has been observed in equimolar ($0.4 \mu\text{M}$) mixtures of enzyme and inhibitor (Vincent & Lazdunski, 1972). We have obtained a comparable result by fluorimetric assay of residual (uninhibited) enzyme in trypsin-Trasytol mixtures, provided that enzyme ($0.073 \mu\text{M}$) and inhibitor were mixed, in appropriate buffer, 5 min before addition of compound I (Fig. 8, open squares). The molecular weight of Trasytol is 6513 (Kassell *et al.*, 1963), but the equivalence point in Fig. 8 is 8000 g/mol of trypsin. We thus assume the Trasytol preparation to be 84% pure. When trypsin was added to buffer containing compound I and Trasytol very little inhibition occurred unless the inhibitor was present in large excess (Fig. 8, closed squares). Similar experiments with acrosin-Trasytol mixtures (Fig. 8) showed that, with enzyme added last, π remained constant while the molar ratio Trasytol/acrosin varied between 0 and 360. However, with prior contact of enzyme and inhibitor, association occurred (Fig. 8, open circles), the equilibrium constant being approx. 10^{-5} M . If the pH was 2–3 during prior contact of either trypsin or acrosin with Trasytol, and release of compound II was initiated by adding the enzyme-inhibitor mixture to buffered compound I, π values were not significantly different from those obtained when either of the enzymes was added to buffer containing compound I and Trasytol.

Discussion

Bender *et al.* (1966) have considered the inherent difficulties in conversion of the velocity of an enzyme-catalysed reaction into a chemically rigorous measure

of quantity of enzyme present. Such calculations call for an absolute standard, namely a completely purified enzyme. This is, in practice, unattainable. Repeatedly crystallized enzymes, particularly proteinases, normally contain impurities and these usually include structurally or chemically modified enzyme. Reaction velocity is itself an uncertain measure of quantity of enzyme present. The ratio of activities of standard and 'unknown' enzyme samples may be influenced by several factors. These include: (1) presence of uncharacterized activators or inhibitors in non-purified enzymes and (2) choice of substrate concentration. For practical reasons the concentration may be below that required for near-saturation conditions and routine determinations of $V_{\max}$ may be deemed too tedious. Further, values of $V_{\max}$ may be affected by activators and inhibitors. As we have shown elsewhere (Brown *et al.*, 1975) the activity of acrosin may be stimulated by inorganic cations, and very low concentrations of an inhibitor present in spermatozoa can influence the effects of cations on acrosin considerably. Such factors tend to invalidate inter-laboratory comparisons. Similarly interspecies comparisons have uncertain value when based on a standardized rate-assay procedure: one that may evoke various degrees of activation among acrosin preparations from different sources.

Most of the above problems may be avoided, or minimized, by assays based on direct titration of active sites. If $[S]_0 \gg K_m$ and ES' is stable, $[P_1]$ is equal to $[E]_0$. The presence of enzyme inhibitors will increase K_m (app.) and the ratio $[S]_0/K_m$ may no longer be high enough to ensure the equivalence of $[P_1]$ and $[E]_0$. However, by making use of the sluggishness of the associations and dissociations of proteinase-inhibitor complexes we have been able to demonstrate the equivalence of acrosin and compound II in the presence of both acrosomal inhibitor and Trasylol and a near-equivalence of trypsin and compound II in the presence of Trasylol (Fig. 8).

A survey of published procedures for active-site titrations revealed that effects of variations in buffer composition, where these involve no change in pH, appear not to have been investigated. There is no presumptive reason why a buffer that gives rise to maximum activity in an overall sequence (eqn. 1), i.e. in a rate assay, should also be most appropriate for the sequence $E + S \rightarrow ES'$ involving a different substrate. A prime requirement for the latter reaction is the stability of ES' , and in this respect the standard buffer containing 1 M-CaCl₂ and dimethyl sulphoxide is superior to barbiturate and barbiturate-CaCl₂ for titration of acrosin with compound I. On the other hand, when the acrosin-catalysed hydrolysis of *N*- α -benzoyl-L-arginine ethyl ester (Brown *et al.*, 1975) was measured in a series of dimethyl sulphoxide-containing buffers of the type specified in Fig. 3, activity reached a maximum with

0.3 M-CaCl₂ and decreased with further rise in CaCl₂ concentration.

Jameson *et al.* (1973) found that at 25°C the burst reaction with α -trypsin and compound I required more than 10 min for completion, whereas with β -trypsin it was complete within the time taken to mix the reagents (5s). We have evidence that the inhibitor-free acrosin consists of more than one active species (Brown *et al.*, 1975), but the time-scale of our titrations rules out the detection of enzyme components with reaction rates comparable with that of β -trypsin.

By an empirical approach we arrived at a fluorimetric titration procedure with three advantages, namely, the burst is rapid, the acyl-enzyme is stabilized, and the post-burst breakdown of compound I is effectively equivalent to the photodecomposition of liberated compound II. This balance depends not only on the buffer composition, and to some extent on $[E]_0/[S]_0$, but also on the intensity of the exciting radiation. One must not therefore assume that with a different instrument a zero post-rate will automatically be obtained in the conditions that we describe. However, with a stabilized acyl enzyme, post-burst rates will be slow enough for reliable extrapolations if instrumental and procedural adjustments fail to produce a negligible net rate of formation of compound II after the burst is complete.

In the wider context of the development of new procedures for titrating hydrolytic enzymes our experience suggests that study of the influences of temperature and of buffer composition (including the addition of activators) may prove fruitful.

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References

- Bender, M. L., Begué-Cantón, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kézdy, F. J., Killheffer, J. V., Marshall, T. H., Miller, C. G., Roeske, R. W. & Stoops, J. K. (1966) *J. Am. Chem. Soc.* **88**, 5890-5913
- Brown, C. R., Andani, Z. & Hartree, E. F. (1975) *Biochem. J.* **149**, 133-146
- Castañeda-Agulló, M. & del Castillo, L. M. (1959) *J. Gen. Physiol.* **42**, 617-633
- Chase, T. & Shaw, E. (1970) *Methods Enzymol.* **19**, 20-27
- Gutfreund, H. & Sturtevant, J. M. (1956) *Biochem. J.* **63**, 656-661
- Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A. & Elmore, D. T. (1973) *Biochem. J.* **131**, 107-117
- Kassell, B., Radicevic, M., Berlow, S., Peanasky, P. J. & Laskowski, M. (1963) *J. Biol. Chem.* **238**, 3274-3279
- Schiessler, H., Fritz, H., Arnhold, M., Fink, E. & Tschesche, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1638-1645
- Vincent, J. P. & Lazdunski, M. (1972) *Biochemistry* **11**, 2967-2977