Titration of the Active Sites in α-Chymotrypsin Solutions with 5-Nitro-1,2-benzoxathiole 2,2-Dioxide

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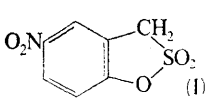
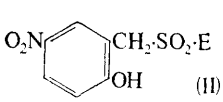
A NUMBER of reagents react stoicheiometrically with the proteolytic enzyme α-chymotrypsin (CT) at its active site, some giving appreciable alteration in the absorption spectrum of the solution. If the turnover of the reagent is sufficiently slow, then the change in the spectrum can be used as a technique for "titrating" CT solutions, *i.e.* determining the molar concentration of the active sites. The usual reagent is *N-trans*-cinnamoylimidazole (CI),[1] which is convenient for titration near pH 5 but *ca.* pH 7—8 titrations are more difficult because of the relatively rapid turnover rate. The rapidity of the deacylation step and/or a relatively slow acylation renders other reagents (*e.g. p*-nitrophenyl acetate) less effective titrants in the higher pH region. Here we describe an accurate method for titrating CT solutions over the pH range 5—7·5 using the single reagent, 5-nitro-1,2-benzoxathiole 2,2-dioxide (I).



(I)



(II)

This compound reacts rapidly stoicheiometrically with CT to give the enzyme sulphonylated at the active site (II).[2] This decomposes to give the active enzyme and products but the half-life even under the most rapid desulphonylation conditions is greater than 15 min. These characteristics coupled with the release of a *p*-nitrophenol chromophore upon sulphonylation make the reagent useful as a titrating agent.

N-trans-Cinnamoylimidazole (CI)[1] had m.p. 133—134°. 1,2-Benzoxathiole 2,2-dioxide[3] was nitrated to give the sultone (I),[4] m.p. 150—151° (EtOH). No decomposition of stock solutions of the reagents in reagent-grade acetonitrile was observed under the storage conditions employed. CT was a thrice crystallized Worthington product. A 30 mg./ml. solution in 0·10M-acetate buffer at pH 5·05 served as a stock solution. pH Values reported were based on a Bates primary standard 7·41 buffer.[5] To avoid spontaneous hydrolysis of the sultone, the buffers were acetate at pH 5 and water-soluble amines at the higher pH's.

Titration with CI was by the method of Schonbaum *et al.*[1] at 335·00 mμ. Extinction coefficients at this wavelength were determined using the same instruments, Cary 14 and Cary 15 spectrophotometers, as used for the titrations.

For titrations with the sultone (I) 100 μl of CT stock solution (~$1{\cdot}0 \times 10^{-3}$ M) was added to 3·00 ml of the desired buffer in a spectrophotometer cuvette. The absorbance A_1 of this solution at the appropriate wavelength served as a baseline. Then 50 μl of the sultone stock solution (~$3{\cdot}0 \times 10^{-3}$ M) was added with stirring. The new absorbance of the solution, A_2, was determined by extrapolation back to the time of sultone addition. The molarity of active enzyme in the stock solution, M_E is given by equation (1)

$$M_E = \frac{A_2 - A_1 - (E_S M_S/63)}{(\epsilon_{ES} - \epsilon_S)\ 10/315} \qquad (1)$$

in which the use of 1 cm. pathlength cells is assumed, ϵ_{ES} and ϵ_S are extinction coefficients of the sulphonyl enzyme and sultone, respectively,

and M_S is the molarity of the sultone stock solution, which is known from the preparation. ϵ_{ES} may be found by adding a known volume of the sultone stock solution to buffer in a cuvette at the desired pH containing a molar excess of enzyme. From the absorbance change ΔA, ϵ_{ES} is calculated by use of equation (2) (the sulphonyl enzyme, (II) will be the only coloured species in the solution).

$$\epsilon_{ES} = \Delta A/(M_S \times \text{dilution factor}) \quad (2)$$

ϵ_S is determined similarly, but with buffer which does not contain enzyme. At pH 5·05 equation (3) with absorbance measured at 321 mμ, was used

$$M_E = (A_2 - A_1 - 33{\cdot}22\,M_S)/149{\cdot}7 \quad (3)$$

At neutral pH values the general equation (4) was used with absorbance measured at 391 mμ. At this wavelength sultone absorbance is no longer significant.

$$M_E = 31{\cdot}5\,(A_2 - A_1)/\epsilon_{ES} \quad (4)$$

Values of the extinction coefficient of the sulphonyl enzyme over a considerable pH range at 391 mμ are given in Table 1. Measured extinction coefficients may differ slightly with different instruments. It is necessary when titrating near neutrality to measure the final pH of the solution to 0·01 unit accuracy so that values of ϵ_{ES} may be found from Table 1.† Alternatively, ϵ_{ES} may be determined as at pH 5.

Duplicate titrations with the sultone agree to $\pm 1\%$, a precision comparable to that possible with CI. The rate of formation of the respective enzyme–substrate covalent intermediates at pH 5

TABLE 1

Extinction coefficient of sulphonyl enzyme (II) *at* $\lambda = 391$ mμ

pH	ϵ_{ES}	pH	ϵ_{ES}
6·37	3,492	7·62	9,276
6·55	4,300	7·76	9,734
6·87	5,849	7·95	10,720
7·15	7,246	8·29	11,850
7·41	8,315	8·71	12,960

is similar. However, the extinction coefficient change at 321 mμ upon release of the nitrophenol chromophore in (II) is only about half that obtained with CI at 335 mμ, so CI remains the reagent of choice at pH 5. The greater stability of the sulphonyl enzyme intermediate near pH 7 makes the sultone a superior reagent in this pH range.

The development of active site titration procedures is of importance in assessing enzyme chemistry quantitatively.[6] The sultone (I), has proved useful for measuring the rate of decrease in active-site concentration of CT solutions during modification by irreversible inhibitors near pH 7.

TABLE 2

Reagent	pH	Wavelength	Buffer	Enzyme conc.[a,b] E_0 10^4M
CI	5·05	335·00 mμ	0·10 M-acetate	9·87[c]
	5·05	335·00 mμ	0·10 M-acetate	9·96[c]
	6·23	335·00 mμ	0·08 M-lutidine(NO_3^-)	9·85
Sultone (I)	5·05	321·00 mμ	0·10 M-acetate	9·44[d]
	6·84	391·00 mμ	0·08 M-lutidine(NO_3^-)	9·61
	7·48	391·00 mμ	0·10 M-Tris(NO_3^-)	9·58

[a] Average of duplicate determinations. [b] The difference between enzyme concentration determined using CI and the sultone seems too small to warrant any interpretation. [c] These titrations at pH 5·05 were performed a week apart on a CT solution stored in the refrigerator at 2°. [d] Average of triplicate determinations.

The concentration of a given enzyme stock solution has been determined using CI and the sultone (I) as titrating agents at several pH values. The results are tabulated in Table 2.

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† We have found ϵ_{ES} to be independent of the buffer systems employed.

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