USE OF SUBSTRATES WITH FLUORESCENT DONOR AND ACCEPTOR CHROMOPHORES FOR THE KINETIC ASSAY OF HYDROLASES

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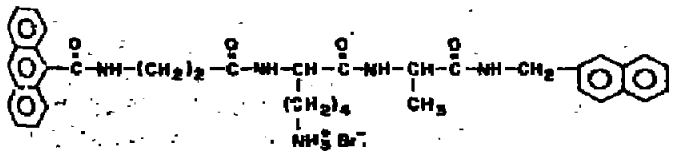
1. Introduction

We wish to present a new method for the kinetic study and quantitative assay of hydrolytic enzymes. The method is based on the interruption of non-radiative energy transfer between two chromophores attached to a substrate molecule. Enzymic cleavage is followed, upon excitation of the donor, by monitoring either the increase in fluorescence of the donor or the decrease in the fluorescence of the acceptor.

Non-radiative energy transfer is a phenomenon in which an excited fluorescent chromophore (the donor) transfers its excitation energy to another chromophore (the acceptor). This transfer results in quenching of the fluorescence of the donor and appearance of the characteristic fluorescence of the acceptor, although the latter is not excited directly. As shown by Förster [1] the yield of transfer depends, among other factors, on the extent of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and decreases with the sixth power of the distance between the chromophores. Separation distances allowing efficient transfer in known donor–acceptor pairs range up to 50 Å [2–4].



Formula I

For compounds in which both chromophores are attached to the same molecule, energy transfer at high dilution will be exclusively intramolecular. Enzymic cleavage of such compounds, resulting in the separation of the donor and the acceptor moieties by diffusion, should obviously lead to a drop in the yield of energy transfer. This effect can be used to follow enzymic activity by monitoring either the enhancement of fluorescence of the donor or the reduction in fluorescence of the acceptor upon excitation of the donor.

As an illustration to the method we have studied the tryptic digestion of several peptides blocked by the donor 2-naphthyl methyl amide and the acceptor anthracene-9-carbonyl amide. Fig. 1 depicts the absorption spectrum of anthracene-9-carbonyl-β-alanyl–lysyl–alanyl-2-naphthyl methyl amide hydrobromide (I) and the emission spectra of acetyl–lysyl–lysyl-2-naphthyl methyl amide di-hydro-chloride (II) and anthracene-9-carbonyl-β-alanyl lysyl methyl ester hydrobromide (III). The absorption spectrum of I is an exact superposition of the absorption spectra of II and III; at the longer wavelengths (315–400 nm) it is due exclusively to the anthracene moiety, whereas at the shorter wavelengths (270–290 nm) it is mainly due to the naphthalene moiety. Fig. 1 shows that there is a considerable overlap between the emission band of the naphthalene donor and the absorption band of the anthracene acceptor. A high yield of energy transfer in compound I should therefore be expected. This is clearly shown in fig. 2, where the emission spectrum of peptide I, due to excitation at 284 nm, is presented. Though at this wavelength the absorption is due almost completely to the naphthalene moiety, its emission is strongly quenched

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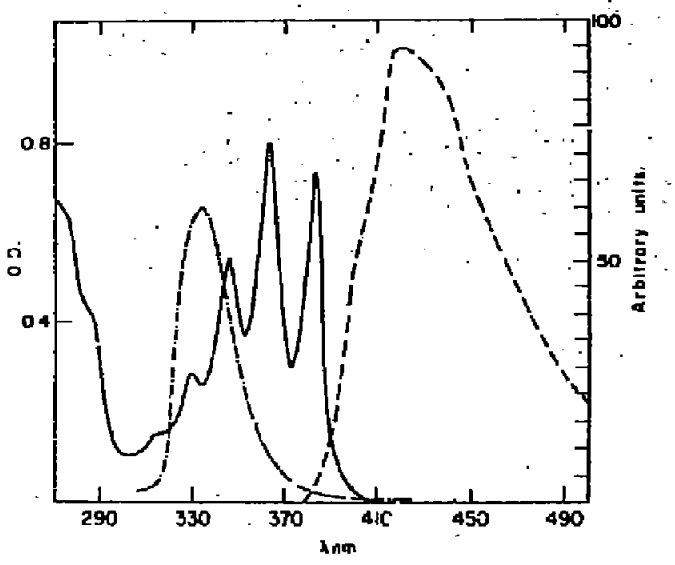

Fig. 1. Absorption spectrum of anthracene-9-carbonyl-β-alanyl lysyl alanyl-2-naphthyl methyl amide (I) (——); emission spectrum of *N*-acetyl lysyl lysyl naphthyl methyl amide dihydrochloride (II) excited at 284 nm (– – – –); emission spectrum of anthracene-9-carbonyl-β-alanyl lysyl methyl ester hydrobromide (III) excited at 363 nm (— – —). Compounds I, II and III were dissolved in 0.02 M Tris-HCl buffer pH 8.7 containing 0.2% methanol. Measurements were carried out at 25°. The emission spectra of II and III are corrected to the same molar concentration. The left ordinate gives optical density due to absorption. The right ordinate gives the intensity of fluorescence in arbitrary units.

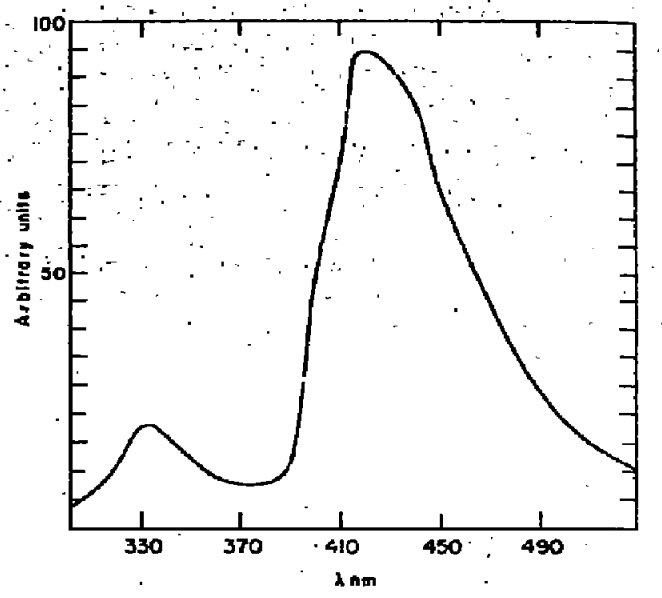

Fig. 2. The emission spectrum of anthracene-9-carbonyl-β-alanyl lysyl alanyl-2-naphthyl methyl amide hydrobromide (I), dissolved in 0.02 M Tris-HCl buffer containing 0.2% methanol, due to excitation at 284 nm. The spectrum is corrected to give the same intensity of fluorescence for the anthracene moiety as that presented in fig. 1. The ordinate represents intensity of fluorescence in arbitrary units.

whereas the anthracene moiety fluoresces as though excited directly.

2. Materials and methods

The synthesis of the following compounds is described elsewhere [5]: anthracene-9-carbonyl-β-alanine, 2-naphthyl methyl amine hydrochloride, anthracene-9-carbonyl alanyl lysyl methyl ester hydrobromide, acetyl lysyl lysyl-2-naphthyl methyl amide, anthracene-9-carbonyl-β-alanyl lysyl alanyl-2-naphthyl methyl amide hydrobromide (I), anthracene-9-carbonyl-β-alanyl $(lysyl)_n$-2-naphthyl methyl amide hydrochloride [n = 2(IV), 3(V), or 4(VI)]. Most of the peptides mentioned above were prepared using *N*-hydroxysuccinimide esters [6] or insoluble polymeric active esters [7]; the three homologous lysine peptide derivatives (IV, V and VI) were prepared by initiating the polymerization of the *N*-carboxyanhydride of ε-benzyloxycarbonyl-lysine with 2-naphthyl methyl amine, blocking the free α-amino groups with anthracene-9-carbonyl-β-alanine, decarbobenzoxylation, and fractionation on a CMC-column. The purity of all the synthesized compounds was ascertained by thin-layer chromatography, elementary analysis, amino acid analysis, high-voltage electrophoresis and vapor phase chromatography.

Fluorograde methanol was purchased from Hartman Ledon Company, Philadelphia. All other reagents were commercial preparations of the highest purity available. Crystalline porcine trypsin Novo (batch No. X630806) was a gift from Novo Industri A/S, Copenhagen. Enzyme concentrations were measured by the absorbance at 280 nm using a Zeiss spectrophotometer, taking $E_{1\%} = 13.1$.

Absorption spectra were obtained using a Cary 15 spectrophotometer. Emission spectra were measured with an Aminco-Keirs spectrofluorometer (American Instrument Co.). High voltage paper electrophoresis was carried out at pH 3.5 (pyridine–acetate buffer) on carboxymethyl cellulose paper. Titrimetric

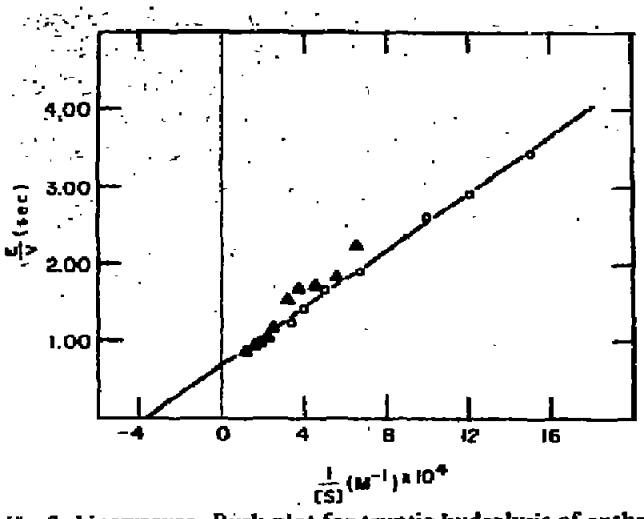

Fig. 3. Lineweaver–Burk plot for tryptic hydrolysis of anthracene-9-carbonyl-β-alanyl lysyl alanyl-2-naphthylmethyl amide (I). The fluorometric assay (o-o-o) was carried out in 0.02 M Tris-HCl buffer containing 0.2% methanol, pH 8.7, at 25°, reaction volume 2.0 ml, concentration range 10^{-6}–10^{-5} M; porcine trypsin concentration 1.5×10^{-8} M. pH-stat titration (▲-▲-▲) was carried out at pH 8.7, 25°, 0.2 M KCl, containing 0.2% methanol, reaction volume 5.0 ml, concentration range 1.5×10^{-5}–8×10^{-5} M; porcine trypsin concentration 9×10^{-8} M. The values of $K_m = 2.7 \times 10^{-5}$ M and $k_{cat} = 1.4$ sec^{-1} were derived from the fluorescence data.

measurements of enzymic activity were performed using a Radiometer pH-stat type PHM 26/TTT11/SBR_2/$TTA3_1$. The steady state kinetic parameters were computed from initial rates over a suitable range of substrate concentration; the Lineweaver–Burk plots being evaluated by the least squares method.

3. Results and discussion

Tryptic digestion of the peptides I, IV, V and VI was followed by monitoring the increase in the fluorescence of the naphthalene moiety at 340 nm upon excitation at 280 nm. The difference between the fluorescence intensity at 340 nm of the exhaustive enzymic digests and the corresponding intact substrates was used as reference (100% hydrolysis). The fluorescence intensity of the final digests was in all of the cases studied equal to that of alanyl-2-naphthyl methyl amide hydrochloride at an equimolar concentration. Fig. 3 shows a Lineweaver–Burk plot of tryptic digestion of compound I as determined by the fluorescence technique described above. For

Table 1
Steady state constants for initial bond hydrolysis by porcine trypsin in the series A–$(Lys)_n$–N(n = 2, 3 and 4)

	K_m (M^{-1})	k_{cat} (sec^{-1})	$C = k_{cat}/K_m$ (M^{-1} sec^{-1})
A–$(Lys)_2$–N	4.1×10^4	29.2	1.20×10^6
A–$(Lys)_3$–N	6.6×10^4	38.0	2.52×10^6
A–$(Lys)_4$–N	3.7×10^5	13.1	4.95×10^6

A = Anthracene-9-carbonyl-β-alanine; N = 2-naphthyl methyl amide. The hydrolysis was monitored by recording the increase in fluorescence at 340 nm, due to excitation at 280 nm. Solutions were made up to 2.0 ml in 0.02 M Tris-HCl buffer pH 8.7, containing up to 10% methanol, 25°. Conc. ranges were 1.10^{-5}–3.10^{-5} M. Porcine trypsin concentration 8×10^{-9}–1.5×10^{-8} M.

comparison the corresponding data derived by the pH-stat assay are included. The fluorescence technique employed yields reliable data at relatively low substrate concentrations (3×10^{-6}–3×10^{-5} M) at which no reliable data could be readily obtained by the pH-stat method. At relatively high substrate concentrations (2×10^{-5}–8×10^{-5} M), corresponding to optical density $A_{340} > 0.1$, on the other hand, the pH-stat method was found to be the method of choice. In this range of substrate concentration the intensity of fluorescence is not proportional to concentration. Finally it should be noted that the straight line going through the fluorescence data in the Lineweaver–Burk plot practically coincides with that going via the pH-stat data. Initial rates were found to be proportional to the enzyme concentration when checked at a concentration range of 3×10^{-8}–1.5×10^{-7} M.

As an additional example to the method of energy transfer interruption we studied the tryptic hydrolysis of the three homologous lysine derivatives IV, V and VI. These peptide derivatives, which have the same absorption spectra as peptide I, undergo tryptic scissions at different sites as established by high voltage electrophoresis of the reaction mixture. Therefore, the rate of occurrence of the first enzymic cleavage cannot be determined by conventional recording methods. However, it is the initial scission which leads to a drop in the yield of energy transfer, regardless of the site of cleavage. Table 1 presents the steady state kinetic parameters thus obtained for the tryptic hydrolysis of the oligolysine derivatives.

Usual spectroscopic assays of hydrolytic enzymes are based on a change in a spectral property due to enzymic splitting off of a chromophoric group. The involvement of the chromophore in the cleavage reaction may complicate the spectral assay and often limits the study of the kinetics of enzymic hydrolysis to substrates possessing susceptible bonds not found in nature. In comparison, the energy transfer interruption method seems to possess the following advantages. Suitable donor–acceptor pairs can be introduced into the substrate molecule at chosen intervals, so as not to participate directly in the enzymic reaction. Hence, changes in energy transfer can reflect directly the rate of cleavage of the susceptible bonds. Great versatility is provided for preparing potential substrates – specially designed for specific research problems concerning different enzymes. The method can be applied not only to endopeptidases, as described here, but also to some exopeptidases. However, in such cases peptide derivatives with a terminal aromatic amino acid containing a corresponding partner for energy transfer should be used. As a matter of fact, during the last phase of this work we were informed about such a study by Latt et al. [8]. An additional advantage of the method is its high sensitivity. Since it is based on fluorometric measurements one can work with substrate concentrations as low as 10^{-6} M. Such low concentrations are particularly desirable when low K_m values are to be determined. By suitable adjustment of the fluorospectrophotometer [9], the method can be applied even to insoluble enzymes, membrane particles, and so on.

Finally it is pertinent to note that the fluorescence quantum yield of some chromophores (e. g. anthracene) is sensitive to changes in microenvironment or to chemical substitution. This might complicate the interpretation of the energy transfer interruption data. This drawback may be overcome, however, by judicious selection of a second chromophore (e. g. naphthyl methyl amide) whose quantum yields does not change as a result of the hydrolysis of the chromophore substrate.

Acknowledgement

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