DIRECT SPECTROPHOTOMETRIC MEASUREMENT OF THE PEPTIDE BOND: APPLICATION TO THE DETERMINATION OF ACYLASE I

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INTRODUCTION

An examination of the absorption spectra of peptides and proteins suggests that their hydrolysis or formation might be followed spectrophotometrically under the proper contitions. A spectrophotometric method would be useful for the study of proteolytic enzymes and protein syntheses.

It has been known for some time that the amide linkage involving an α-amino acid absorbs radiant energy in the region of the spectrum below 240 mμ[1]. The absorption spectrum of peptides involving nearly all amino acids has been determined for the region of 200 to 240 mμ[2,3,4]. The absorption coefficients of simple N-acylamino acids, amino acid amides, and the more complex peptides are greater in this region than those of their component amino acids. The intensity of absorption of these amino acid derivatives is proportional to the quantity of amide bonds present, provided that corrections are made for the absorption of the component amino acids. At the initiation of the present investigation, it became apparent that the technique of differential spectrophotometry could be used to detect the amide linkage directly. This, in turn, made it possible to determine enzymic activity based on the rate of change in the difference spectrum between the amino acid derivative and its expected hydrolysis products during hydrolysis of the derivative.

The enzyme in this investigation was renal acylase I, which hydrolyzes N-acetyl-L-amino acid to form amino acid. The study has provided a sensitive and convenient assay of acylase activity.

Since it is hoped that this simple system will serve as a model for more complex proteolytic enzyme determinations, the present report considers in detail certain spectroscopic aspects which are well known to spectroscopists but might be overlooked by the biochemist who is interested in a protease determination.

EXPERIMENTAL

Materials

Acetyl-L-methionine was prepared as suggested in the general procedure outlined by BIRNBAUM *et al.*[5]. Analysis gave 7.33% N, $[\alpha]_D$ —20.3° (4% in H_2O), m.p. 103.5–104.5° C, as against reported[5] 7.4% N, $[\alpha]_D$ —20.1° (4% in H_2O), m.p. 104° C.

Commercial L-methionine (Schwarz) was used.

Renal acylase I was prepared in our laboratory in collaboration with Dr. E. RAPKIN by the method of BIRNBAUM *et al.*[5]. Acylase IA (cobalt activated) was prepared by the method of

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MARSHALL[6] from partially purified acylase I. For one part of the study a purified sample of renal acylase I was generously supplied by J. P. GREENSTEIN and co-workers.

Apparatus

The Beckman Model DU spectrophotometer with photomultiplier was used with 1 cm quartz cells. The cell compartment was kept at constant temperature by circulating water through the thermospacers in the cell compartment. The standard lamp housing supplied with the instrument contained coils which permitted cooling.

Solutions

0.025 *M* solutions of acetyl-L-methionine and L-methionine were prepared in 0.1 *M* phosphate buffer at pH 7. Both agents in solution appeared stable on incubation at 37° C for several hours. For long-term storage the solutions, in small lots, were kept frozen.

Method

In a typical assay, 3 ml of methionine solution is placed in a 1 cm cuvette and 3 ml of acetylmethionine solution in a matching cuvette. The cuvettes are permitted to equilibrate at 25° C in the cell compartment. The instrument is balanced against the methionine solution at 238 mμ and a slit width of ≦ 0.3 mm with the photomultiplier at position 4. Then the optical density of the acetyl-L-methionine solution is determined. At zero time, 0.01 ml of acylase solution is added with a micropipette to the substrate-containing solution and the mixture is stirred for a few seconds. Optical density measurements are made immediately and at intervals of 30 sec until several uniform increments in optical density are observed between 90 and 60% of the total initial optical density.

Calculations

Specific acylase activity (A) expressed as micromoles of substrate hydrolyzed per h/mg protein (enzyme) is calculated from the formula

$$A = \frac{\Delta D \cdot 60 \cdot 1000}{E_m \cdot \text{mg protein/ml}}$$

where ΔD is optical density change per minute and E_m is the molar extinction coefficient of the amide bond of acetyl-L-methionine (24 at 238 mμ and 25° C). Protein concentrations for acylase (mg protein/ml) were determined from the optical densities of their solutions at 280 mμ. The extinction coefficient of highest purity acylase (14.3% N) was 0.996/cm/mg/ml. The activity values reported are the average of three experiments.

RESULTS

In the first phase of this study, spectral differences in the far ultraviolet were easily demonstrated between methionine and acetylmethionine when their optical densities were determined against water[1]. Optical density differences between solutions of acetylmethionine and methionine at identical concentrations are apparently caused by this amide linkage. At a concentration of 0.01 *M*, a maximum in amide spectrum occurred at 228 mμ. However, at higher concentrations the maximum decreased and shifted to higher wave lengths (Fig. 1). These observations could not be accepted as representing spectral differences between these two materials. SAIDEL, GOLDFARB AND KALT[7] attribute a similar abnormal absorption to stray radiation, whose effect

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is exaggerated by the use of differential spectrophotometry[8]. This effect, which becomes appreciable at lower wave lengths or higher concentrations, should be corrected for[7]; however, for this assay it was found practical to avoid the region of serious stray radiation. The amount of stray radiation varies from one instrument to another but usually is small enough to cause no serious interference above 230 mμ[6].

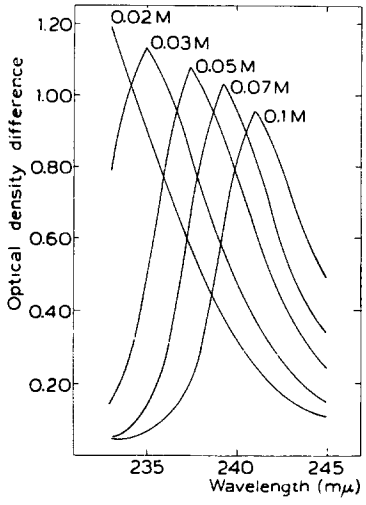

Fig. 1. Difference spectra of identical concentrations of acetylmethionine and methionine at 25° C, pH 7 (0.1 *M* phosphate).

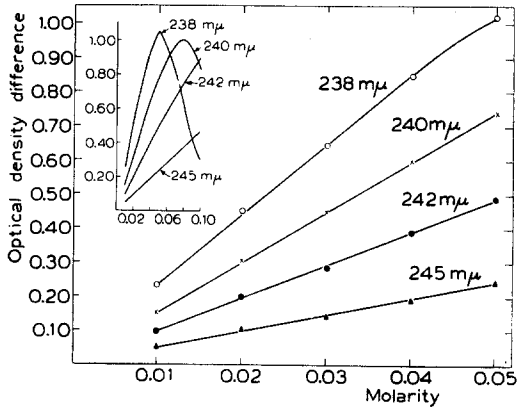

Fig. 2. Optical density difference between acetylmethionine and methionine at various concentrations at 25° C, pH 7 (0.1 *M* phosphate). The insert is a direct comparison of identical concentrations of acetylmethionine and methionine. The larger plot represents various molarities of acetyl methionine (ordinate) plus methionine to total 0.05 *M* "methionine" compared with methionine of 0.05 *M* concentration.

As seen in Fig. 2, the ascending portion of the plot of concentration *vs.* optical density difference between acetylmethionine and methionine follows Beer's law. The wave length of 238 mμ was chosen for this assay because it was above the region where stray radiation causes serious interference[7] at a workable substrate concentration (0.025 *M*) and in the region where the slope of the optical density difference —molarity curve (Fig. 2) was comparatively steep. Although the plot at 238 mμ is linear up to 0.04 *M*, the concentration of 0.025 *M* was selected to allow for the additional absorption of the enzyme.

With the establishment of these conditions, it was possible to evaluate this method. Values obtained by this new method agree well with values obtained by the more tedious VAN SLYKE ninhydrin-CO_2 method[8]. (See Table I.) These samples range from the crudest water extract of porcine kidney (homogenate) to the more highly purified acetone fractions. A linear relationship exists between initial velocity and enzyme concentration for a range of 20 to 100 μg of highly purified acylase per ml (Fig. 3).

This method can also be used for kinetic studies of acylase. The Michaelis-Menten constant for acetyl-L-methionine was found to be $5.2 \cdot 10^{-3}$ *M* at 25° C. R. D. MARSHALL *et al.*[6] have reported the constant to be $3.7 \cdot 10^{-3}$ *M* at 37° C.

The temperature chosen to do most of this work was 25° C because it is readily maintained. Since most of the data reported in the literature are at 37°, a factor of 1.6 was calculated from the data in Fig. 3 for conversion of the 25° C rate to the 37° rate.

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TABLE I

COMPARISON OF ACYLASE ASSAY METHODS

Analyst *Buffer* *Method*	*A* *Veronal-acetate* VAN SLYKE	*B* *Veronal-acetate* VAN SLYKE	*C* *Phosphate* VAN SLYKE	*D* *Phosphate* *Spectrophotometric*
Homogenate		100*	92	91
Partially purified**		963		978
Partially purified cobalt treated		1140		1120
Highly purified***	2393	2390		2302

* Activities are expressed in μmoles substrate hydrolyzed per h/mg protein (enzyme) at 25°.
** Armour's technical grade.
*** From J. P. GREENSTEIN, who also supplied assay A.

In the process of determining the effect of change in temperature on the enzyme, an optical density difference (0.1) was found to exist between methionine (0.025 *M*) at 25° C and 37° C. There exists also an optical density difference between samples of acetylmethionine at different temperatures which is considerably greater than that exhibited by methionine. The optical density difference between methionine and acetylmethionine of identical concentrations at 238 mμ increases rapidly as the temperature rises (Fig. 4). With constant-temperature controls used on the cell holder and the lamp, this temperature effect proved to be no problem.

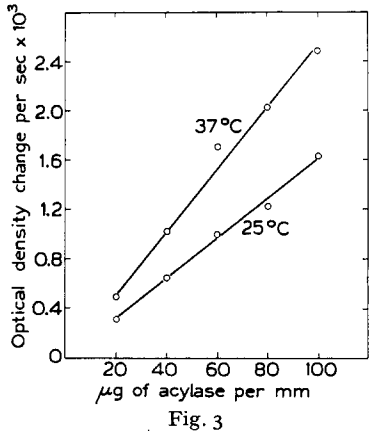

Fig. 3

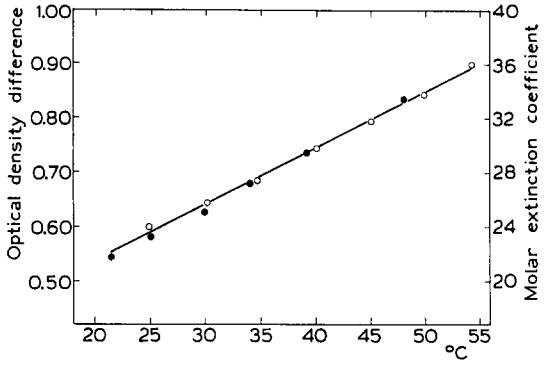

Fig. 4

Fig. 3. Optical density change per second at 238 mμ due to the action of various concentrations of acylase on acetyl-L-methionine at pH 7 (0.1 *M* phosphate).

Fig. 4. Optical density difference between 0.025 *M* L-methionine and 0.025 *M* acetyl-L-methionine at various temperatures at pH 7 (0.1 *M* phosphate).

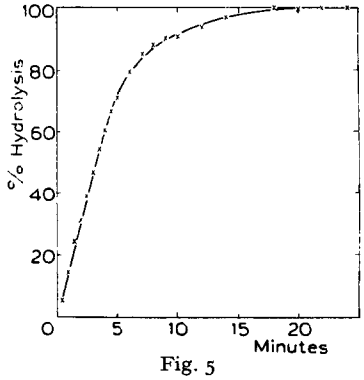

Fig. 5

Fig. 5. The course of hydrolysis of 0.025 *M* acetyl-L-methionine with acylase at 25° C, pH 7 (0.1 *M* phosphate) as followed by the spectrophotometric method.

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DISCUSSION

This direct photometric method provides a means for the determination of enzymic activity as well as for the convenient study of enzyme kinetics. The assay is sensitive, simple, and rapid. Even with partially purified enzyme preparations, a satisfactory determination can be obtained in two to five minutes. Despite the fact that the most highly purified acylase available is not a pure enzyme, as little as 10 μg is required for a determination. Apparent zero order kinetics hold true up to 40% hydrolysis of the total substrate (Fig. 5), providing ample opportunity for a satisfactory rate determination.

Other factors in the acylase system favor the development of this assay. Relatively low substrate concentrations can be used, because of the affinity of acylase for substrate*. The rate of hydrolysis is relatively great, so that a short time is sufficient for an assay. The substrate and the enzyme are both stable.

Other proteolytic enzymes may be determined by a method similar to the one described. The absorption of acetylated methionine is only one-third greater than that of the free amino acid at 238 mμ. Many di- and tripeptides exhibit even greater spectral differences. Since the average contribution of a peptide bond to the absorption of a protein is fairly constant[10,11] it is possible that even proteins might be used as substrate for proteolytic determinations.

In certain instances it may be desirable to use shorter wave lengths than 230 mμ. However, the large amount of stray radiation in this region makes it impossible to use the ordinary spectrophotometer without making corrections. This problem may be solved by modification of instruments.

SUMMARY

A sensitive, continuous, and direct spectrophotometric assay for acylase has been presented and the possibility of extending this method to other proteases is discussed.

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* A shorter light path (narrower cells) would permit the use of higher substrate and enzyme concentrations.