BBA 66013

DETERMINATION OF THE STEREOSPECIFIC HYDROLYTIC ACTION OF PEPSIN BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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(Received July 18th, 1969)

SUMMARY

Nuclear magnetic resonance spectroscopy has been used to follow the enzymatic hydrolysis of *N*-Ac–DL-Phe–L-Tyr by pepsin The rate of reaction and the stereospecificity of the enzyme were determined with an accuracy of $\pm 3\%$

INTRODUCTION

Once the specificity of a biological agent toward amino acid derivatives of known optical configuration has been established, its action towards amino acids of unknown stereochemistry can be predicted with some confidence One of the important limitations in the use of such biological procedures is that the homogeneity and the specificity of some enzyme preparations are in doubt In order to evaluate the purity of a biological agent before use, it is often necessary to check its performance with a model substrate

The susceptibility of racemic substrates to enzymatic attack has been followed by a sequence of absorbance, colorimetry or other suitable techniques to measure the rate of hydrolysis, followed by an optical purity determination of the product[1,2] We have recently demonstrated that the chromatographic separation of diastereoisomers can be used to determine the rate and the stereospecific action of leucine aminopeptidase[3] and acylase[4] in one experimental run, but even here some time-consuming chemical manipulations were required We have now used nuclear magnetic resonance spectroscopy (NMR) to verify the stereospecific action of pepsin on a model substrate Since no physical separation of the diastereoisomeric mixture is required for analysis by NMR, the kinetic constants and the optical purity of the products can be determined without any chemical modifications being required before analysis

MATERIALS AND METHODS

Materials

N-Ac–L-Phe–L-Tyr and *N*-Ac–D-Phe–L-Tyr were obtained from the Mann

Research Lab , N Y Fresh substrate solutions were prepared according to BAKER[5,6] and used immediately 3-times crystallized pepsin, (EC 3 4 4 1) derived from hog stomach[7], was supplied by Mann Research Lab , N Y Dimethyl sulfoxide-d_6 (99 5%) and ^2H_2O (99 8%) were obtained from Diaprep, Incorporated, Atlanta and trifluoroacetic acid was supplied by Eastman Organic Chemicals, Rochester

Equipment

Varian A-60 nuclear magnetic resonance spectrometer, Virtis lyophilizer and Clinical centrifuge (International Equipment Co)

Method

The incubation mixtures were made up by dissolving *N*-Ac–L-Phe–L-Tyr (212 mg, 5 μM) or *N*-Ac–L-Phe–L-Tyr (170 mg, 4 μM) and *N*-Ac–D-Phe–L-Tyr (170 mg, 4 μM) in turn in 0 5 M NaOH, 0 5 M HCl, and 0 5 M NaCl and adding pepsin (75 mg $\equiv$ 0 100 mg pepsin N per ml and 60 mg $\equiv$ 0 080 mg pepsin N per ml, respectively) The total reaction volume was 120 ml and the pH of the solution was 3 The hydrolysis was followed by removing aliquots (20 ml) with a pipette every 15 min and by transferring them to a Virtis lyophilizing flask (50 ml) The flasks were immediately cooled in dry ice to stop the reaction, and the water was then removed by freeze-drying The residue was triturated with dimethyl sulfoxide-d_6 (1 ml) and the suspension transferred to a centrifuge tube After centrifugation, the supernatant was pipetted into an NMR tube (pressure-cap seal) ready for NMR analysis The spectra were obtained on a Varian A-60 spectrometer, and for quantitative analysis peak areas were calculated from "half-height measurements" (height $\times$ width at $^1/_2$ height) or "vertex measurements" The latter method involves dropping a perpendicular line from the intersecting point of the two peaks onto the integration curve and measuring the heights corresponding to the two peak areas on the integration curve (Fig 1)

RESULTS

1 Assignment of NMR resonance peaks

The determination of the degree of enzymatic hydrolysis depends on an accurate peak area measurement of the signals due to the methyl resonances of the substrate at 104 4 Hz and the hydrolysis product (*N*-Ac–L-Phe) at 108 0 Hz The ratio of peak area (*N*-Ac–L-Phe) peak area (*N*-Ac–L-Phe–L-Tyr + *N*-Ac–L-Phe) corresponds to the percent hydrolysis of the substrate NMR analysis of a synthetic mixture of substrate and product corresponding to a 53% hydrolysis value, yielded a figure of 56% by the half-height method and a value of 54% by the vertex procedure (Fig 1A) The signal due to the protons of the phenyl ring at 433 7 Hz (*N*-Ac–L-Phe–L-Tyr) and 434 9 Hz (*N*-Ac–L-Phe) may also be used to determine the degree of hydrolysis, but since the degree of resolution of the two peaks is less, an accurate area measurement is much more difficult

A stereochemical analysis of the substrate in solution is possible because the proton resonances of the phenyl ring for *N*-Ac–L-Phe–L-Tyr is at 433 7 Hz and that for *N*-Ac–D-Phe–L-Tyr is at 430 9 Hz Since the signal for the product, *N*-Ac–L-Phe, is at 434 9 Hz, the phenyl resonance peaks can be used to determine the optical

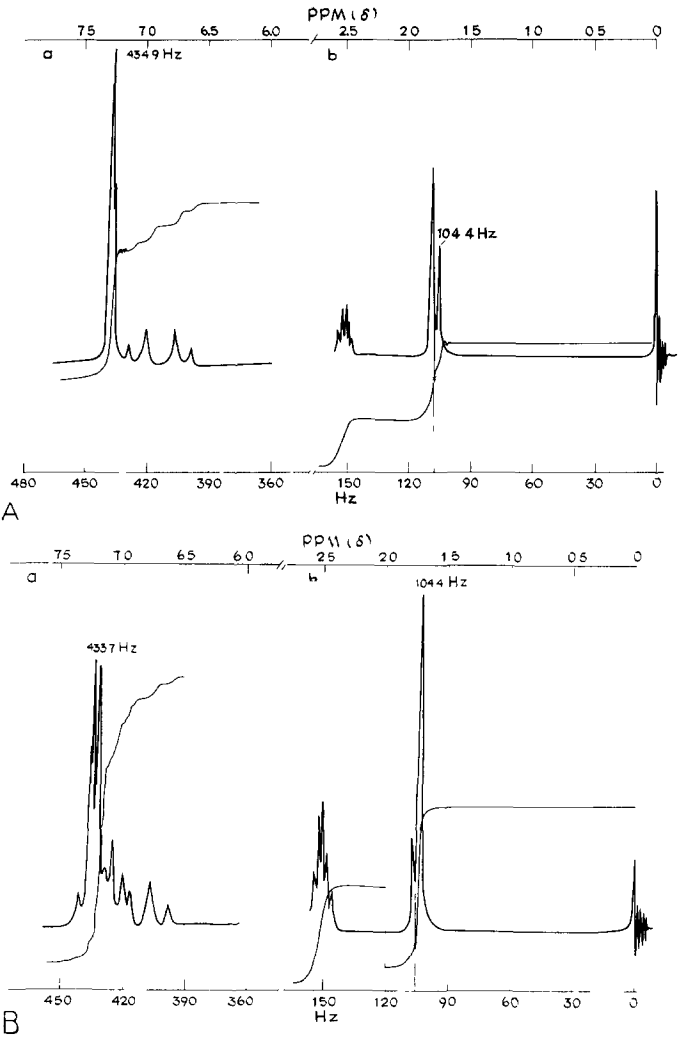

Fig 1 A NMR spectrum of a synthetic mixture of substrate (*N*-Ac-L-Phe-L-Tyr) and product (*N*-Ac-L-Phe) corresponding to a 53% hydrolysis value The solution contained *N*-Ac-L-Phe-L-Tyr (27 mg 0 127 mM) and *N*-Ac-L-Phe (18 mg, 0 142 mM) dissolved in dimethyl sulfoxide-d_6 (0 4 ml) and ^2H_2O (0 2 ml) and acidified with trifluoroacetic acid to pH 3 B NMR spectrum of a synthetic mixture of racemic substrate (*N*-Ac-DL-Phe-L-Tyr) and product (*N*-Ac L-Phe) The solution contained *N*-Ac-L-Phe (3 8 mg 30 μM), *N*-Ac-L-Phe-L-Tyr (13 3 mg 60 μM), and *N*-Ac-D-Phe-L-Tyr (20 0 mg, 90 μM) dissolved in dimethyl sulfoxide-d_6 (0 5 ml) and ^2H_2O (0 1 ml) and acidified with trifluoroacetic acid (1 μl) to pH 6

purity of the substrate during enzymatic hydrolysis An NMR analysis of a synthetic mixture of racemic substrate and product corresponding to 33 3% product showed the ratio of the (*N*-Ac-L-Phe-L-Tyr + *N*-Ac-L-Phe) (*N*-Ac-D-Phe-L-Tyr) peak areas to be 1 098 (1 1 LL DL predicted) using the vertex method of area determination (Fig 1B)

TABLE I

PEPSIN-CATALYZED HYDROLYSIS OF *N*-Ac-L-Phe-L-Tyr

Spectra were run under the following conditions on a Varian A-60 probe temperature = 36°, filter bandwidth = 1 Hz, R F field = 0.2 mgauss, sweep time = 500 sec, sweep width = 500 Hz, sweep offset = 0.0 Hz, spectral amplitude = 65, integral amplitude = 80 (with spectral amplitude attenuated to 10) Curvature and Y field gradients were adjusted until a resolution of 0.3 Hz or better was obtained, with a minimum signal-to-noise ratio of 10.06 Tetramethylsilane was used as an internal standard Peak area determinations were made on expanded spectra with methyl resonances at 104.0, 104.4 and 108.0 Hz, the dimethyl sulfoxide-d_6 resonances at 150.5 Hz, and the phenyl resonances at 430.9, 433.7, 434.9 Hz Instrument settings were filter bandwidth = 0.4 Hz, sweep time = 250 sec, sweep width = 100 Hz, spectral amplitude = 80, integral amplitude = 40 (with spectral amplitude attenuated to 10), minimum signal-to-noise ratio = 14.5, sweep offset adjusted appropriately All other instrument settings were as before In addition particular attention was paid to proper detector phase and detector zero adjustment, since it was found to be essential to have precisely symmetrical peaks and properly adjusted integration curves to attain reproducible results

Sample	*Time (min)*	*% Hydrolysis*		*(Substrate + product area) / Internal standard area**
		Half-height method	*Vertex method*	
1	15	15.3	23.7	0.659
2	30	32.2	36.4	0.652
3	45	38.5	40.3	0.649
4	60	45.1	48.0	0.591
5	75	47.3	53.4	0.542
6	90	50.0	56.9	0.600
				Mean = 0.616
				S.E. = 0.0118
				Coefficient of variation = 7.49%

* Since the dimethyl sulfoxide-d_6 is only 99.5% pure, the residual protons, averaging one per methyl group, interact with the two deuterium atoms on each methyl group producing a 1:3:5:3:1 quintet at 150.5 Hz This can then be used as an internal standard Since this column is statistically constant it follows that the molar concentration of substrate *plus* product remains constant throughout the reaction which indicates the reliability of the NMR procedure

2 *Pepsin-catalyzed hydrolysis*

The results of a pepsin-catalyzed hydrolysis run using *N*-Ac-L-Phe-L-Tyr as the substrate are summarized in Table I It is worth noting that the rate of hydrolysis as determined by the NMR method is in excellent agreement with the results obtained by BAKER[5] (50% hydrolysis of the substrate after 60 min) It is also significant that *N*-Ac-D-Phe-L-Tyr does not inhibit the reaction, since *N*-Ac-L-Phe-L-Tyr in the racemic mixture is hydrolyzed at the same rate (Table II) This experiment also demonstrates the stereospecific action of pepsin, since the concentration of *N*-Ac-D-Phe-L-Tyr remains the same during the course of the enzymatic hydrolysis (Table II)

DISCUSSION

These experiments show the utility of NMR for studying enzymatic hydrolysis On the basis of data obtained from synthetic mixtures, an accuracy of $\pm 3\%$ is possible by peak area measurement determined by the vertex method By selection of appropriate enzyme–substrate systems ($K_m \geqslant 10^{-1}$), the Michaelis–Menten con-

TABLE II

PEPSIN-CATALYZED HYDROLYSIS OF *N*-Ac–DL-Phe–L-Tyr

Sample	*Time (min)*	*% Hydrolysis (LL) (Vertex method)*	*% Hydrolysis (DL)*	DL *area* / *Internal standard area**
1	15	35 7	—	—
2	30	38 7 ± 0 7	—	—
3	45	48 6 ± 2 7	0 0	0 439
4	60	49 6 ± 0 9	0 0	0 426
5	75	55 9 ± 0 4	0 0	0 430
6	90	58 5 ± 1 0	0 0	0 426
				Mean = 0 430
				S E = 0 003
				Coefficient of variation = 1 4%

* The proportion of the DL diastereoisomer was determined by dividing the area under the DL peak (vertex determination) at 430 9 Hz by the internal standard (vertex determination, dimethyl sulfoxide-d_6 quintet at 150 5 Hz) If this ratio is constant over the course of hydrolysis as these results show, then hydrolysis of the diastereoisomer is 0%

stant, (K_m), other kinetic parameters, and the stereospecificity can be determined While the above enzymatic hydrolyses were carried out with 4–5 μM substrate solutions in the buffer systems described above, we had to concentrate the solutions by freeze-drying before the NMR measurements, to improve the signal-to-noise ratio However, the use of a time averaging computer in conjunction with the A-60 spectrometer, obviates the need for such a concentrated solution

By choosing suitable reaction conditions such as (1) a ^2H_2O-soluble substrate, (2) a characteristic NMR signal for both substrate and product which can be followed during the reaction, (3) a rate of hydrolysis not to exceed 50% per h with $K_m \geq 10^{-1}$, it will be possible to obtain the kinetic parameters while the reaction is actually in progress Furthermore, by using a variable temperature probe the MNR technique could also be employed to study the effect of temperature on the reaction rate

ACKNOWLEDGMENTS

This work was supported by a fellowship to E J by the Norwegian Cancer Society and by National Aeronautics and Space Administration Grant NGR-05-020-004

We particularly want to thank Dr Lois Durham, Director, NMR Lab, Stanford Chemistry Department for her continued advice and consultation throughout this project

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