Biochimica et Biophysica Acta, 485 (1977) 402—405

BBA 68292

THE EFFECT OF SAMPLE VISCOSITY ON THE DEACYLATION OF *p*-FLUORO-*trans*-CINNAMOYLCHYMOTRYPSIN

J.T. GERIG and R.S. McLEOD

Department of Chemistry, University of California, Santa Barbara, Santa Barbara, Calif. 93106 (U.S.A.)

(Received June 9th, 1977)

Summary

The deacylation of *p*-fluoro-*trans*-cinnamoylchymotrypsin at pH 5.0 in the presence of variable amounts of polyvinylpyrrolidone has been examined. The sample viscosity at the highest polymer concentration used was over 200 times greater than that of pure buffer but no effects on the deacylation kinetics were observed.

Introduction

An in vivo enzymatic reaction takes place in a milieu that is considerably more complex than that used to examine the reaction in a laboratory setting. A variety of large and small molecules are present in vivo with the result that the viscosity of the medium in which the catalyzed reaction takes place is substantially from that of aqueous buffers. A number of investigators have explored the influence of neutral polymers such as dextran, polyethyleneglycol and polyvinylpyrrolidone on enzymatic systems and it has been observed that these polymeric materials can increase [1—4] or decrease [3] activity, often in non-linear ways.

Some of the possible effects of polymers on enzymatic reactions have been discussed by Laurent [3]. These include changes of the chemical potentials (activity coefficients) of the enzyme and substrate(s), restriction of molecular diffusion and the formation of protein · polymer or polymer · substrate complexes. Previous studies of polymer effects on enzyme action have been analyzed in terms of Michaelis-Menten type kinetic schemes that have not permitted dissection of the observed effects into the influence of polymer on each discrete step in the enzyme reaction mechanism. Clear demonstrations of the various possible polymer effects mentioned above have thus been elusive.

We have reported previously a study of the deacylation of various fluorine-

substituted cinnamoylchymotrypsins [5]. These structures are isolatable intermediates in the α-chymotrypsin-catalyzed hydrolysis of cinnamoyl esters and amides and are stable enough to make experimentally feasible a study of polymer effects on a single step in the chymotryptic mechanism, namely deacylation. This report describes the effect of polyvinylpyrrolidone on this reaction.

Materials

p-Nitrophenyl-*p*-fluoro-*trans*-cinnamate was prepared as described previously [5]. The enzyme sample used was a Worthington thrice-recrystallized product. Buffers were 0.05 M in acetate and contained 0.1 M KCl. Matheson spectroquality acetonitrile was used without further purification.

Polyvinylpyrrolidine (Type NP-K90, average molecular weight 360 000) was obtained commercially from GAF Corp. as a pale yellow powder. The material was dialyzed against 1 mM HCl and then distilled water, followed by lyophilization to afford a fluffy, white material. The dialysis tubing (Union Carbide) had a nominal molecular weight cut-off of 14 000.

N-Methylpyrrolidone (Aldrich) was distilled before use. Glycerol (Mallinckrodt) was distilled in vacuo from calcium hybride before use.

Methods

The rate of deacylation of *p*-fluoro-*trans*-cinnamoylchymotrypsin was followed by the same procedure as described earlier except that the temperature was maintained at 30.0° C instead of 30.2° C. Polymer was added to the buffered enzyme solution; an aliquot of a stock *p*-nitrophenyl ester solution in acetonitrile was added to initiate the reaction such that the concentration of acetonitrile in the final reaction mixture was 6.25% by volume. With the very viscous solutions the mixing process introduced air bubbles. These were cleared by allowing the cuvettes to stand in a vacuum desiccator which was carefully evacuated so as to avoid foaming of the samples. Treatment of the absorbance vs. time data was by the same method used previously.

Viscosity of the polymer solutions were measured over the concentration range 0 to 112 mg/ml using a falling ball viscometer (Gilmont No. 1, purchased from Cole-Parmer). The viscometer containing a polymer solution was submerged in a constant temperature bath at 30.0° C; each drop time was measured at least three times to obtain an average value used in the calculation of the viscosity. Maximum deviation from the mean within these measurements did not exceed 5%. Aqueous glycerol solutions were used to calibrate the viscometer [6].

Results

Deacylation of *p*-fluoro-*trans*-cinnamoylchymotrypsin was determined at pH 5 in the presence of polyvinylpyrrolidone with the polymer concentration ranging up to 103 mg/ml. As a control for the effect of organic material in this system, several experiments were run with *N*-methylpyrrolidone present at a level comparable to the amount of polymer used. Table I summarizes the results.

A small increase in sample pH was observed when polymer or *N*-methyl-

TABLE I

DEACYLATION OF *p*-FLUORO-*trans*-CINNAMOYLCHYMOTRYPSIN [a]

Added material	Concn. (M) [b]	pH [c]	η (cP) [d]	$k_\psi \times 10^4$ (s^{-1}) (observed) [e]	$k_\psi \times 10^4$ (s^{-1}) (calculated) [f]
None	0	5.01	0.8	2.42	2.32
Polyvinylpyrrolidine					
0.56 mg/ml	0.005	5.01	0.9	2.49	2.32
2.6 mg/ml	0.023	5.02	1.5	2.49	2.37
5.5 mg/ml	0.2	5.03	5.5	2.58	2.42
103 mg/ml	0.93	5.11	175.0	2.97	2.86
N-Methylpyrrolidone	0.25	5.05	≈0.9	2.58	2.52
N-Methylpyrrolidone	1.01	5.23	≈0.9	3.54	3.70

[a] At 30.0°C; acetate buffer (0.05 M) containing 0.1 M KCl and 6.25% acetonitrile.
[b] Molar concentration of *N*-alkylpyrrolidone groups contributed by added polyvinylpyrrolidone or *N*-methylpyrrolidone.
[c] Measured pH of reaction mixture.
[d] Viscosity of reaction mixture, measured at 30°C.
[e] Observed first-order rate constant for deacylation.
[f] Calculated rate constant for deacylation at the observed sample pH, obtained using parameters in ref. 5.

pyrrolidone was added to the reaction mixture. To account for the pH changes on the deacylation rate, the pH-dependence of the reaction in the absence of added organic materials was assumed to apply to the systems containing polymer or NMP. The differences between the observed rate constants and those calculated with the assumed pH-dependence is 5% or less, a variation that is less than the combined estimated experimental uncertainties in the observed and calculated parameters. Thus, neither polyvinylpyrrolidone nor the monomeric model compound, *N*-methylpyrrolidone, have any detectable effect on the rate of deacylation of this enzyme intermediate.

Discussion

The catalytic mechanism for the action of α-chymotrypsin can be represented by Eqn. 1 in which the enzyme (E) and the substrate

$$E + S \rightleftharpoons ES \rightleftharpoons ES' + P_1 \rightleftharpoons EP_2 + P_1 \rightleftharpoons E + P_2 + P_1 \quad (1)$$

combine to form a Michaelis complex (ES). Within this complex, the acyl part of the substrate (P_2) is transferred to the enzyme to give an enzyme-ester (EP_2) and release an amine, thiol or alcohol moiety represented by P_1. The experiments described herein focus on the hydrolysis of EP_2 and are insensitive to previous steps in the mechanism. Our finding that polymer does not influence this reaction step is not surprising as any change in the activity of water induced by polymer is expected to be small. At the highest concentration of polymer used, the reaction mixtures are so viscous that they pour only with difficulty. However, the amount of added organic material is reasonably small, corresponding nominally to approx. 1 mol/liter of *N*-alkylpyrrolidone.

Trón et al. [4] have studied the effect of polyvinylpyrrolidone on the trypsin-catalyzed hydrolysis of *N*-benzoyl-L-arginine ethyl ester. The polymer had a substantial effect on enzyme activity, increasing it in a manner that depended

on polymer concentration and sample temperature. To explain these results an "energy-funnel" model which postulates energy uptake for catalysis at specific sites on the enzyme surface was invoked [7]. Energy accretion from colliding molecules at these sites depends upon a number of parameters including the sample viscosity. Trypsin is structurally very similar to chymotrypsin [8]. Our results suggest that "energy-funnel" effects likely are not influenced by the presence of polyvinylpyrrolidone at the stage of the enzymatic mechanism of trypsin or chymotrypsin in which the acylenzyme is hydrolyzed, but rather, if present at all, must be felt at an earlier point in the catalytic sequence.

Acknowledgements

The authors gratefully acknowledge the support of the National Science Foundation (Grant CHE73-05209). J.T.G. is a PHS Research Career Development Awardee (Grant GM-70373).

References

1 Ceska, M. (1971) Experientia 27, 767—768
2 Ceska, M. (1972) Experientia 28, 146
3 Laurent, T.C. (1971) Eur. J. Biochem. 21, 498—506
4 Trón, L., Somogyi, B., Papp, S. and Novák, M. (1976) Studia Biophys. 60, 157—162
5 Gerig, J.T. and McLeod, R.S. (1975) Can. J. Chem. 53, 513—518
6 Lange's Handbook of Chemistry, 9th edn., p. 1709
7 Somogyi, B. and Damjanovich, S. (1975) J. Theoret. Biol. 51, 393—401
8 Stroud, R.M., Kay, L.M. and Dickerson, R.E. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 125—140