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Short Communications

Studies on *Streptomyces griseus* Protease

IV. Formation of ^{14}C-Acetyl Enzyme of two DFP-Sensitive Components during Reaction with *p*-Nitrophenyl [1-^{14}C]Acetate

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The purification of two DFP-sensitive enzyme components, with esterase activity against *p*-nitrophenyl acetate (PNPA),* from a commercial preparation of *Streptomyces griseus* protease (Pronase, Calbiochem) was recently described from this laboratory.[1] A high degree of purity was accounted for by an incorporation of one mole of phosphorus from DFP per mole of enzyme, and from a test of homogeneity on polyacrylamide gel electrophoresis. These two enzymes, PNPA-hydrolase I and PNPA-hydrolase II, show similarities with chymotrypsin in those respects that they catalyze the hydrolysis of PNPA, and that the primary structure around the DFP-reacting serine residue is the same in all three enzymes.[2] It therefore seemed to be of interest to see whether these two enzymes from Pronase become acetylated during their catalysis, as has been shown for chymotrypsin.[3,4]

PNPA-hydrolases I and II were purified as described before.[1] *p*-Nitrophenyl [1-^{14}C]acetate (NEN Chemicals GmbH, Dreieichenhain, Germany) and unlabelled PNPA (Eastman) were dissolved in 1,4-dioxane at a final concentration of 6×10^{-2} M. The specific radioactivity of the PNPA was then about 5×10^{2} cpm/nmole. DF^{32}P with a specific radioactivity of 2.2×10^{4} cpm/nmole was obtained from the Radiochemical Centre, Amersham, England.

Protein concentration, PNPA-hydrolase activity, radioactivity of ^{14}C and ^{32}P were measured as described before.[1,4]

To determine the amount of active enzyme, the two PNPA-hydrolases were incubated with 6×10^{-4} M DF^{32}P at pH 8.0 for 1 1/2 h at room temperature. Unreacted DFP was removed by chromatography on Sephadex G-50 in 0.1 M Tris-HCl buffer, pH 8.0. The amount of active enzyme was calculated from the amount of radioactivity incorporated under the assumption that all the active enzyme molecules had reacted with the inhibitor.[5] Both PNPA-hydrolases were found to contain 98 % active enzyme.

The acetylation of PNPA-hydrolases I and II were studied with the aid of a rapid-mixing apparatus [6,4] at pH 8.0 in 0.1 M Tris-HCl buffer at final concentrations of 0.05 mM enzyme and 3 mM substrate. The reaction was interrupted after different times by the addition of trichloroacetic acid-silicotungstic acid. The precipitated protein was collected by centrifugation, dissolved in 0.1 M sodium phosphate buffer, pH 8.0 containing 0.5 % sodium lauryl sulfate, and chromatographed on a column of Sephadex G-25 as described before.[4] The chromatographed protein was then analyzed with respect to ^{14}C-radioactivity.

The stability of the ^{14}C-acetyl-enzyme was tested at 50° at pH 5, pH 7, and pH 9 as previously described.[4] At pH 7, only about 5 % of the ^{14}C-acetyl groups were released by this treatment. The corresponding values at pH 5 and 9 were about 25 %. Thus the chromatography at pH 7 could be used for the isolation of the acetyl enzyme.

Fig. 1 shows Lineweaver-Burk plots of the steady state hydrolysis of PNPA by PNPA-hydrolase I (A) and PNPA-hydrolase II (B). The values of apparent K_m calculated from the slope of the straight lines were 0.9×10^{-4} M for PNPA-hydrolase I and 1.3×10^{-4} M for PNPA-hydrolase II. It can also be seen that contrary to the

* Abbreviation: PNPA, *p*-nitrophenyl acetate.

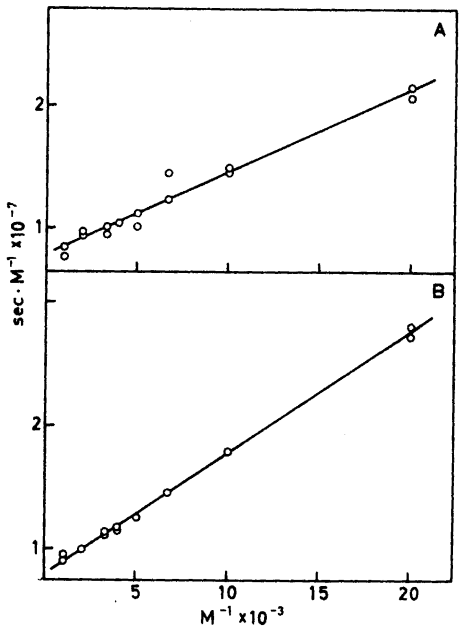

Fig. 1. Lineweaver-Burk plots of the steady-state hydrolysis of *p*-nitrophenyl acetate by PNPA-hydrolase I (A) and PNPA-hydrolase II (B) at 25° in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 % 1,4-dioxane. Enzyme concentrations: A 3.5×10^{-6} M; B 6.4×10^{-6} M.

corresponding plot for the chymotrypsin-catalyzed hydrolysis of PNPA, no evidence for substrate activation was obtained.[4,7,8] The turnover at 3 mM PNPA was found to be approx. 0.04 sec^{-1} for PNPA-hydrolase I and 0.02 sec^{-1} for PNPA-hydrolase II.

Fig. 2 shows the acetylation of PNPA-hydrolase I as a function of time. The maximal degree of acetylation, which was obtained after 15 sec, amounted to about 35 % for PNPA-hydrolase I. The slope of the initial part of the curve then gives an apparent rate constant of the acetylation reaction of about 1 sec^{-1}. The rate of acetylation consequently is large enough to permit the formation of an intermediately acetylated enzyme.

The value for maximal acetylation of PNPA-hydrolase II was about 10 %. Within 200 msec, the acetylation did not exceed 2 %. Therefore, no reliable values of the initial rate of acetylation could be

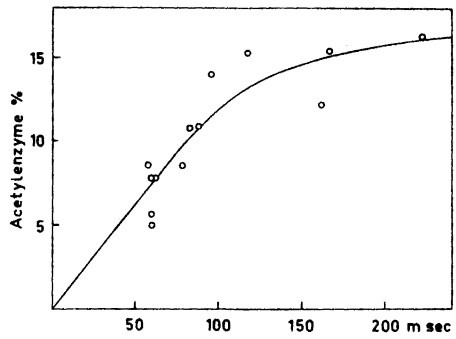

Fig. 2. Initial acetylation of PNPA-hydrolase I by *p*-nitrophenyl acetate at 25° in 0.1 M Tris-HCl buffer, pH 8.0, 5 % 1,4-dioxane. Enzyme concentration: 5×10^{-5} M. Substrate concentration: 3×10^{-3} M.

obtained for PNPA-hydrolase II by the rapid-mixing technique. However, the fact that maximal acetylation was obtained within 15 sec, suggests an intermediate acetylation of PNPA-hydrolase II as well.

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