ACETYLATION OF PRONASE TRYPSIN[1]

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Summary - Reaction of Pronase trypsin with acetic anhydride yielded a homogeneous, active, and stable derivative. This was achieved by including glycerol in the acetylation reaction as previously described. Acetylation resulted in no change in K_m and only a moderate decrease in V_{max} with N^{α}-benzoyl-L-arginine-p-nitroanilide as substrate. As with bovine trypsin the single amino terminal residue was not acetylated. This is in contrast to the other homologous mammalian and microbial enzymes where complete acetylation of amino terminal residues is noted. Thus, a close conformational homology is suggested around the amino-termini of the microbial and mammalian trypsins.

The purification of Pronase trypsin has been described (1-6) with sequence studies demonstrating marked homology with mammalian trypsin (7). The mammalian enzymes of the chymotrypsin family are characterized by an ion-pair formed by the β-carboxyl group of the aspartyl residue adjacent to the reactive serine and the α-amino group of either isoleucine or valine (8-11). This bond is formed after activation of the zymogens when limited tryptic hydrolysis generates the above new amino terminal residues (8,12). The effect of acetylation of the mammalian enzymes on their activity has been studied extensively since acetylation of the α-amino groups would change the nature of the ion pair bond (8,13,14). Because Pronase trypsin and two associated smaller components which are homologous with bovine chymotrypsin have both the specific aspartyl residue next to the reactive serine and also either amino terminal isoleucine or valine, their reaction with acetic anhydride has been of some interest to us. An earlier report described the reaction of the two smaller enzymes (15,16); we now describe the results achieved after acetylation of Pronase trypsin.

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Experimental Procedures

Streptomyces griseus trypsin was purified as before (1,6) from commercial Pronase (lot numbers 900053, 000130 and 101185) obtained from Calbiochem. DFP was obtained from Amersham-Searle Corporation. The activities against Bz-Arg-OEt[2] and Bz-Arg-NHNp were determined by previously described techniques (1,6). Bz-Arg-NHNp was obtained from Protein Research Foundation, Osaka, Japan. L-Valyl-L-valine was obtained from Fox Chemical Co.

The reaction of Pronase trypsin with acetic anhydride, the chromatography procedure, and analysis for purity by gel electrophoresis were done by the techniques applied earlier to the two homologous enzymes (16). The earlier important modification of including glycerol (20% v/v) in the acetylation mixture permitted studies to be carried out with very small amounts of protein. Because acetylated trypsin appeared to autolyze during gel electrophoresis, both native and acetylated enzymes were reacted with DFP before application to the polyacrylamide gels. Twenty µl of protein solution were applied for each run. The amount of protein applied was determined according to the methods of Lowry *et al.* (17) using bovine serum albumin as the standard. Spectra were carried out with a recording Spectrophotometer (Zeiss DMA-21) of native and acetylated proteins in 5 mM sodium acetate - 5 mM calcium acetate (pH 5). The amino terminal residues of the native and acetylated enzymes were examined qualitatively after performic acid oxidation by reaction with dansyl chloride (18). The results were analyzed on the quartered polyamide sheets as described earlier (16,18,19). The identity of each fluorescent spot was confirmed by chromatographic comparisons with dansyl derivatives of standard amino acids and dipeptides. The quantitation of amino terminal residues was determined by the cyanate procedure (21), after the protein had been oxidized by performic acid (22). Chromatography through a Dowex 1-X8 column was

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2. Abbreviations used are: Bz-Arg-OEt, N^{α}-benzoyl-L-arginine ethyl ester; Bz-Arg-NHNp, N^{α}-benzoyl-L-arginine-*p*-nitroanilide; dansyl, dimethylamino-naphthylsulfonyl; EDTA, ethylenediaminetetraacetate.

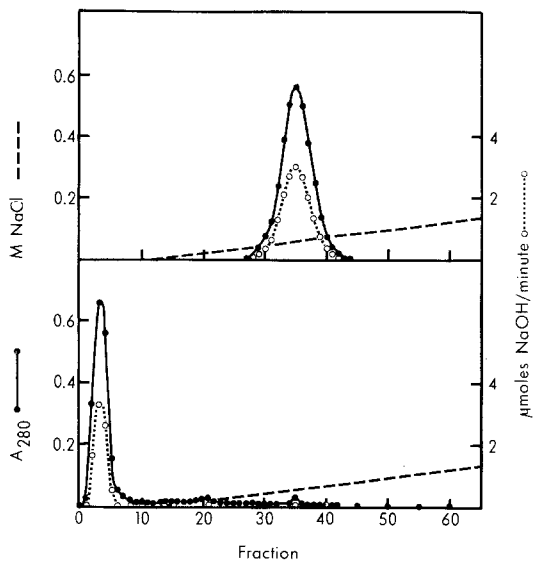

Figure 1. Chromatography through CM-cellulose of native (upper panel) and acetylated (lower panel) S. griseus trypsin; about 10 mg of protein were applied in each run. Fractions of 3.0 ml were collected. The activity against Bz-Arg-OEt is demonstrated; 50 μl aliquots were used for the assays. See text for details.

carried out to remove peptides containing cysteic acid from the hydantoins. The recoveries for amino-terminal residues were calculated using the data of Stark (21), without consideration for losses during chromatography through the Dowex-1 column; therefore, the values reported are probably low.

Because of the apparent low K_m that the microbial enzyme demonstrated toward Bz-Arg-OEt, this substrate could not be applied satisfactorily for accurate determination of kinetic constants. Instead, Bz-Arg-NHNp was utilized; the low concentrations of substrate that were necessary for these studies required the use of cuvets with a 10 cm light path. The enzymatic activity was followed by the change in absorbancy at 405 nm using a Cary 15 recording spectrophotometer; a molar extinction coefficient of 9,620 per cm light path was used for p-nitroaniline at this wavelength (10).

Results

The procedure of acetylation in the presence of glycerol resulted in a completely soluble product. Fig. 1 depicts the patterns of chromatography through CM-cellulose of native and acetylated S. griseus trypsin. Acetylation

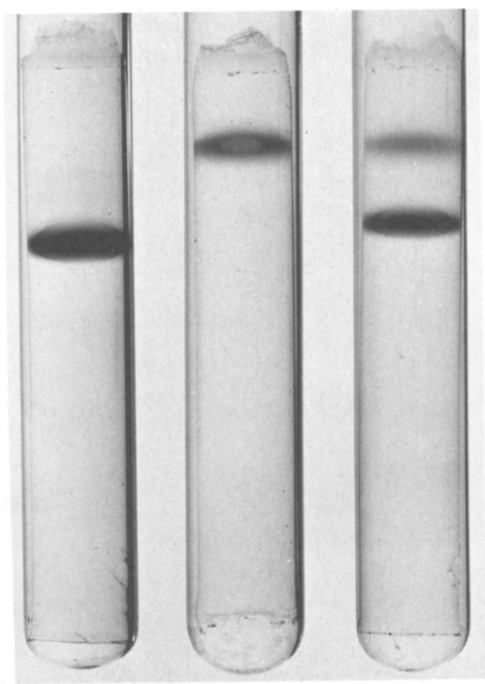

Figure 2. Acrylamide gel electrophoresis of unacetylated and acetylated S. griseus diisopropylphosphoryl-trypsin. Left, unacetylated enzyme, 60 µg; center, acetylated enzyme, 20 µg; right, 30 µg of unacetylated and 10 µg of acetylated enzymes. Migration from top to bottom, cathode at the bottom.

yields material which demonstrated no affinity for the cation exchange column in marked contrast to the native enzyme. Furthermore the derivative appears to be fully active.

The homogeneity of acetylated S. griseus trypsin as demonstrated by acrylamide gel electrophoresis can be seen in Fig. 2. The derivative, as expected, migrates much less rapidly towards the cathode than the native protein. It should be noted that this study extends the previous evidence for homogeneity of the enzyme since it would be distinctly unusual for two or more different proteins to demonstrate parallel migrations in both the native and acetylated states. The extent of acetylation of α-amino groups was determined both qualitatively and quantitatively. Following treatment with dansyl chloride both native and acetylated enzymes demonstrated the presence of dansyl-valine and dansyl-L-valyl-L-valine after acid hydrolysis. The latter derivative results from the well-known observation that the conditions

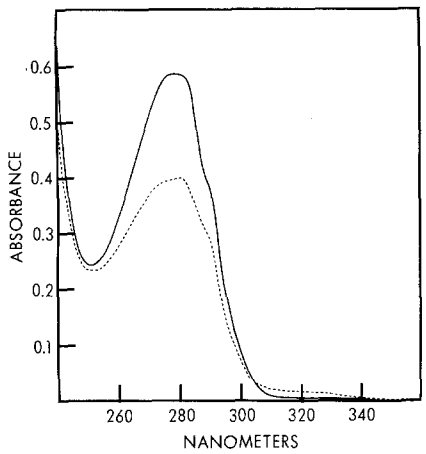

Figure 3. Ultraviolet spectra of native (————, 0.55 mg/ml) and acetylated (·····, 0.40 mg/ml) S. griseus trypsin.

of acid hydrolysis do not permit complete cleavage of bonds in dipeptides with residues which are branched at β carbons if the amino terminus is blocked by a stable and bulky ligand. Since the acetylated protein was determined to be electrophoretically homogeneous, it is clear that there can not be any substantial enzyme fraction which is acetylated at the amino-terminus. This interpretation is strengthened by the quantitative amino-terminal analyses as determined by the cyanate procedure, where the values found for native and acetylated proteins were respectively 0.44 and 0.46 moles of valine per mole of protein.

Fig. 3 depicts the spectra of native and acetylated enzyme. The spectrum of the acetylated enzyme is substantially different from that of the parent protein; the ratio of absorbancies at 280 and 260 nm is lower after acetylation. The spectrum of the derivative did not change after exposure to acetate at pH 5.0 for several days at 4°. This finding is similar to those noted with the acetylated derivatives of the two homologous proteins in Pronase (16). However, the spectral changes of the trypsin component after acetylation are not as marked as those noted with the two smaller proteins. The possibility remains that there may be O-acetylated tyrosine residues which are unusually resistant to cleavage in these enzymes.

Table 1

Kinetic constants for hydrolysis of Bz-Arg-NHNp

Protein	K_m	V_{max}
	mM	sec^{-1}
Native enzyme	0.029	5.2
Acetylated enzyme	0.029	3.0

Studies were carried out at 22° in 10 mM Tris·HCl (pH 8.0) with 5 mM $CaCl_2$. The range of substrate concentrations were from 5.6 to 168 μM and the enzyme concentrations were from 0.8 to 1.0 nM.

The kinetic constants noted in Table 1 reveal no substantial differences between native and acetylated proteins; the Michaelis constants are identical while the maximal velocity of the acetylated enzyme is about 60% of the native enzyme. Very slight differences in kinetic constants were noted in the case of bovine trypsin and its acetylated derivatives (13).

Discussion

The stabilizing effect of glycerol during acetylation reactions with acetic anhydride is demonstrated for the second time in this study with Pronase trypsin. An earlier study showed that glycerol was required during this reaction in order to obtain stable derivatives from two companion homologous Pronase endopeptidases (16). Insoluble and inactive products were noted in that investigation after acetylation in the absence of glycerol. Glycerol permits the generation of excellent yields of stable derivatives, an important consideration when only a scant amount of native protein is available.

The amino-terminal residue is clearly spared from acetylation. This is in marked contrast to the results noted with the two smaller homologous

enzymes present in Pronase (16). In all three microbial cases acetylation does not substantially affect the catalytic activity. The three homologous bovine enzymes (chymotrypsin, elastase, and trypsin) demonstrate a similar pattern of accessibility of α-amino groups to acetic anhydride. The α-amino groups of chymotrypsin and elastase are readily acetylated (8,14), whereas, as in the case of the Pronase enzymes, the α-amino residue of the trypsin component does not react (13). This suggests that the conformational similarity at the amino-termini is very close for these two trypsins, closer than that manifested by each trypsin with respect to companion homologous components. This is remarkable in view of the vastly different biological sources of the two trypsins. It has been shown in denaturant, that bovine trypsin can react with different electrophiles at the α-amino site (24,25). We have been unable to obtain a homogeneous protein with the bacterial enzyme after acetylation in denaturant.

References

1. Vosbeck, K., Chow, K.-F., and Awad, W.M., Jr. (1973) J. Biol. Chem. 248, 6029-6034.
2. Wählby, S. (1968) Biochem. Biophys. Acta 151, 394-401.
3. Trop, M. and Birk, Y. (1968) Biochem. J. 109, 475-476.
4. Narahashi, Y. and Fukunaga, J. (1969) J. Biochem. (Tokyo) 66, 743-745.
5. Jurášek, L., Fackre, D., and Smillie, L.B. (1969) Biochem. Biophys. Res. Commun. 37, 99-105.
6. Awad, W.M., Jr., Soto, A.R., Siegel, S., Skiba, W.E., Bernstrom, G.G., and Ochoa, M.S. (1972) J. Biol. Chem. 247, 4144-4154.
7. Olson, M.O.J., Nagabhushan, N., Dzwiniel, M., Smillie, L.B., and Whitaker, D.R. (1970) Nature 228, 438-442.
8. Oppenheimer, H.L., Labouesse, B., and Hess, G.P. (1966) J. Biol. Chem. 241, 2720-2730.
9. Sigler, P.B., Blow, D.M., Mathews, B.W., and Henderson, R. (1968) J. Mol. Biol. 35, 143-164.
10. Shotton, D.M. and Watson, H.C. (1970) Nature 225, 811-816.
11. Stroud, R.M., Kay, L.M., and Dickerson, R.E. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 125-140.
12. Neurath, H. (1957) Advan. Protein Chem. 12, 319-386.
13. Labouesse, J. and Gervais, M. (1967) Eur. J. Biochem. 2, 215-223.
14. Kaplan, H. and Dugas, H. (1969) Biochem. Biophys. Res. Commun. 34, 681-685.
15. Siegel, S., Brady, A.H., and Awad, W.M., Jr. (1972) J. Biol. Chem. 247, 4155-4159.
16. Siegel, S. and Awad, W.M., Jr. (1973) J. Biol. Chem. 248, 3233-3240.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
18. Gros, C. and Labouesse, B. (1969) Eur. J. Biochem. 7, 463-470.
19. Woods, K.R. and Wang, K.-T. (1967) Biochim. Biophys. Acta 133, 369-370.

20. Awad, W.M., Jr., Ochoa, M.S., and Toomey, T.P. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 2561-2565.
21. Stark, G.W. (1967) Methods Enzymol. 11, 125-138.
22. Hirs, C.H.W. (1967) Methods Enzymol. 11, 197-199.
23. Pfleiderer, G. (1970) Methods Enzymol. 19, 514-521.
24. Chevalier, J., Yon, J., and Labouesse, J. (1969) Biochim. Biophys. Acta. 181, 73-81.
25. Robinson, N.C., Neurath, H., and Walsh, K.A. (1973) Biochemistry 12, 420-426.
26. Russin, D.J., Floyd, B.F., Toomey, T.P., Brady, A.H., and Awad, W.M., Jr. (1974) J. Biol. Chem. 249, in press.