Lysostaphin Endopeptidase-Catalysed Transpeptidation Reactions of the Imino-Transfer Type

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The glycylglycine endopeptidase in lysostaphin has been found capable of catalysing both hydrolysis and transpeptidation reactions when acting on glycyl peptides. The ability of the enzyme to utilize dansyldiglycine (5-dimethylaminonaphthalene-1-sulphonylglycylglycine) as an acceptor molecule in transpeptidation reactions, although it is incapable of hydrolysing the peptide bond in this compound, indicates the enzyme must be capable of forming the equivalent of an imino-enzyme intermediate during the catalytic process.

Lysostaphin is a protein preparation obtained from the culture filtrate of a bacterium in the genus *Staphylococcus* (*Staphylococcus staphylolyticus*, N.R.R.L. B2628). This preparation is a powerful lytic agent for *Staphylococcus aureus*, but not for a large number of other micro-organisms (Schindler & Schuhardt, 1964; Cropp & Harrison, 1964; Schindler, 1966). Lysostaphin contains three components capable of acting on bacterial cell-wall peptidoglycan: a glycylglycine endopeptidase, a hexosaminidase (endo- β -N-acetylglucosaminidase) and an N-acetylmuramyl-L-alanine amidase (Browder *et al.*, 1965; Wadström & Vesterberg, 1970). These three components can be separated by gel filtration or by isoelectric focusing (Wadström & Vesterberg, 1970).

The glycylglycine endopeptidase in lysostaphin is a zinc-metalloenzyme of mol.wt. 25000 (Trayer & Buckley, 1970). The endopeptidase is the component in lysostaphin that lyses *S. aureus* cells. This enzyme hydrolyses glycylglycine bonds in the polyglycine cross-bridges occurring between glycopeptide chains in the cell wall of *S. aureus* (Browder *et al.*, 1965). These polyglycine cross-bridges appear to be a genus-specific characteristic of staphylococci and presumably explain the limited range of bacteriolytic action of the endopeptidase (Tipper & Strominger, 1966; Strominger & Ghuysen, 1967). Browder *et al.* (1965) reported that the endopeptidase was capable of hydrolysing glycyl peptides such as pentaglycine. These investigators did not report the method used to detect hydrolysis products, nor the products formed from these hydrolysis reactions. The present paper reports our findings in an investigation of the action of the endopeptidase on glycyl peptides.

Experimental

Materials

Lysostaphin (207 units/mg), tetraglycine and hexaglycine were obtained from Schwarz-Mann, Orangeburg, NY, U.S.A. Purified fractions from lysostaphin were kindly supplied by Dr. P. A. Tavormina, Mead Johnson Research Center, Evansville, IN, U.S.A. Polyglycine was purchased from Miles Laboratories, Elkhart, IN, U.S.A. All other amino acids and peptides and dansyl* chloride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All chemicals were used without further purification. T.l.c. media (Gelman ITLC media, type S) were purchased from Gelman Instrument Co., Ann Arbor, MI, U.S.A.

Methods

Action of lysostaphin and its purified components on glycyl peptides. Glycyl peptides to be tested (10–25 μ mol of each) were incubated for 8–10 h at room temperature (24°C) with 100 μ g of lysostaphin in 1 ml of Tris/NaCl buffer (0.05 M-Tris/HCl buffer, pH 7.5, made up to 1.0 M with NaCl). The contents of the tubes were then dried *in vacuo* with a Rotary Evapo-Mix (Buchler Instruments Co., Fort Lee, NJ, U.S.A.). Controls were treated similarly, except for the addition of lysostaphin. The products from the enzymic reactions and the unchanged substrate controls were converted into fluorescent derivatives by resuspending the contents of the tubes in 0.5 ml of

* Abbreviation: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

0.2M-NaHCO₃ and adding an equal volume of dansyl chloride in acetone (2.5mg/ml). After 2h the contents of the tubes were dried *in vacuo* as described above and resuspended in 1ml of Tris/NaCl buffer. The dansyl derivatives of the controls and reaction products were chromatographed on Gelman type S ITLC media by using 2-methylbutan-2-ol saturated with 0.1M-potassium hydrogen phthalate buffer, pH6.0, as the solvent system. Control and experimental solutions (5 μ l each) were applied to the t.l.c. sheet with separate disposable micro-pipettes. After chromatography the chromatograms were viewed under u.v. light and the products identified by comparison of their R_F values with those of known dansyl-peptides and dansylglycine run on the same sheets.

Similar experiments also were run with purified glycyglycine endopeptidase, hexosaminidase and amidase from lysostaphin.

Action of lysostaphin on dansylglycyl peptides. Solutions (1ml) of previously dansylated glycyl peptides in Tris/NaCl buffer (10 μ mol/ml) were made to react with 100 μ g of lysostaphin for 12h at room temperature. Samples (5 μ l) of the reaction mixtures were chromatographed by using the chromatographic procedure described above, and the products identified by their R_F values.

Utilization of dansylglycine and dansyldiglycine as acceptor molecules in transpeptidation reactions. In attempts to determine the type of mechanism used by the endopeptidase in catalysing transpeptidation reactions, dansylglycine and dansyldiglycine were used as potential acceptors for the transpeptidation reactions. In these experiments, 20 μ mol of triglycine

was made to react with 100 μ g of lysostaphin in the presence of either 5 μ mol of dansylglycine or 5 μ mol of dansyldiglycine in 1ml of Tris/NaCl buffer. At zero time, after $\frac{1}{2}$ h and every subsequent hour during the reaction period, 5 μ l portions were removed from the reaction mixtures and applied to a sheet. The chromatograms were developed as described above and viewed under u.v. light to detect the presence of fluorescent transpeptidation products.

Results and Discussion

Action of lysostaphin and its purified components on glycyl peptides

Of the purified lysostaphin components, only the endopeptidase was found to be capable of acting on the glycyl peptides. For this reason the lysostaphin preparation used in all subsequent studies did not require further purification of the endopeptidase. Fig. 1 shows tracings of chromatograms of dansylated products from the reaction of several glycyl peptides with lysostaphin. These chromatograms demonstrate that the endopeptidase in lysostaphin catalyses both hydrolysis and transpeptidation reactions when acting on these glycyl peptides. We found the tendency for transpeptidation reactions could be enhanced by increasing the substrate concentration.

Action of lysostaphin on dansylglycyl peptides

Fig. 2 depicts a tracing from a chromatogram showing the products from the reaction of dansylated glycyl peptides with lysostaphin. Dansyldiglycine

Fig. 1. Tracings of chromatograms showing the action of lysostaphin endopeptidase on glycyl peptides

The reaction products and controls were chromatographed as their fluorescent dansyl derivatives on Gelman type S ITLC media with 2-methylbutan-2-ol saturated with potassium hydrogen phthalate buffer, pH6.0, as the solvent system. The reaction mixtures contained 25 μ mol of each glycyl peptide and 100 μ g of lysostaphin in 1 ml of Tris/NaCl buffer, pH7.5, and were incubated at room temperature for 8h before reaction with dansyl chloride. Abbreviations: (Gly)₂, diglycine; (Gly)₃, triglycine; etc. '+E' indicates that enzyme was added to the reaction mixture.

Fig. 2. Tracing from chromatogram showing action of lysostaphin endopeptidase on dansylated glycyl peptides. The reaction mixtures contained 10 μ mol of each dansylglycyl peptide and 100 μ g of lysostaphin in 1 ml of Tris/NaCl buffer, pH 7.5, and were made to react together for 12 h at room temperature. Details of the chromatographic procedure are given in the text. Abbreviations: -(Gly)₂, -diglycine; etc.

Fig. 3. Tracing of chromatogram demonstrating imino-type transfer in transpeptidation reactions catalysed by lysostaphin endopeptidase.

The reaction mixture contained 5 μ mol of dansyl-diglycine, 20 μ mol of triglycine and 100 μ g of lysostaphin in 1 ml of Tris/NaCl buffer, pH 7.5. The reaction mixture was incubated at room temperature, and 5 μ l portions were removed at various time intervals and spotted on the t.l.c. sheet. Details of the chromatographic system are given in the text.

was found to be resistant to hydrolysis by the endopeptidase, possibly because the large dansyl group sterically hinders proper orientation of this compound with the binding residues in the active site. Larger dansylated glycyl peptides, however, were hydrolysed to smaller peptides, but no further than to dansyldiglycine.

Utilization of dansylglycine and dansyldiglycine as acceptor molecules in transpeptidation reactions

The ability of the lysostaphin endopeptidase to catalyse transpeptidation reactions illustrates the general capacity found in most peptidases and proteinases to utilize either water or another peptide as an acceptor molecule after cleavage of a susceptible peptide bond. Many proteolytic enzymes, including trypsin, chymotrypsin, papain, ficin and pepsin, possess this ability (Fruton, 1971). There are two possible mechanisms for transpeptidation reactions. In one type of mechanism an acyl-enzyme intermediate is formed, and the acyl portion of the peptide substrate is subsequently passed to an amino acceptor to complete the reaction:

This mechanism is reported to exist in all proteinases

studied to date, with the possible exception of pepsin (Newmann *et al.*, 1959; Fruton, 1971, 1976).

The second possible mechanism for transpeptidation involves the formation of an imino-enzyme intermediate and the transfer of the amine portion of the peptide substrate to a carboxylic acceptor:

This is the mechanism that may be used by pepsin in transpeptidation reactions, although this theory is questionable (Silver & Stoddard, 1973; Fruton, 1974, 1976).

Fig. 3 shows a tracing from a chromatogram of the products from the reaction of triglycine and lysostaphin in the presence of dansyldiglycine. Although the enzyme cannot hydrolyse the peptide bond in the dansyldiglycine, it can use this compound as an acceptor in transpeptidation reactions, as is evidenced by the appearance of fluorescent transpeptidation products after 1 h reaction time. Since the endopeptidase is incapable of hydrolysing the peptide bond in dansyldiglycine, the only possible mechanism that can explain the production of fluorescent transpeptidation products under these conditions would involve the formation of the equivalent of an imino-enzyme intermediate in the transpeptidation reactions:

An acyl-enzyme intermediate could not produce fluorescent transpeptidation products under these conditions, owing to the blocking of the *N*-terminus of the dansyldiglycine by the dansyl group:

Gly-Gly-CO-E + dansyl-Gly-Gly (no reaction)

Dansylglycine was found to be incapable of acting as an acceptor molecule in transpeptidation reactions. If this molecule can bind in the active site, it apparently is too far removed from the catalytic residues to act as an acceptor in transpeptidation.

The ability of a peptidase or proteinase to catalyse transpeptidation reactions implies there is an ordered sequential release of products from the active site of the enzyme. The evidence from the present study suggests that lysostaphin endopeptidase can form the equivalent of an imino-enzyme intermediate in its catalytic mechanism, in which the carboxylic product is released before the amino product. Whether there is actually a covalent imino-enzyme intermediate has not as yet been determined. It is also uncertain at this time whether the enzyme is capable, under certain conditions, of releasing the amino product first, thereby also producing the equivalent of an acyl-enzyme intermediate.

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