Action of human liver cathepsin B on the oxidized insulin B chain

Malcolm J. MCKAY,* Margaret K. OFFERMANN,*‡ Alan J. BARRETT† and Judith S. BOND*§

*Department of Biochemistry, Virginia Commonwealth University, Richmond, VA 23298, U.S.A., and

†Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, U.K.

(Received 23 March 1983/Accepted 27 April 1983)

The lysosomal cysteine proteinase cathepsin B (from human liver) was tested for its peptide-bond specificity against the oxidized B-chain of insulin. Sixteen peptide degradation products were separated by high-pressure liquid chromatography and thin-layer chromatography and were analysed for their amino acid content and *N*-terminal amino acid residue. Five major and six minor cleavage sites were identified; the major cleavage sites were Gln₍₄₎–His₍₅₎, Ser₍₉₎–His₍₁₀₎, Glu₍₁₃₎–Ala₍₁₄₎, Tyr₍₁₆₎–Leu₍₁₇₎ and Gly₍₂₃₎–Phe₍₂₄₎. The findings indicate that human cathepsin B has a broad specificity, with no clearly defined requirement for any particular amino acid residues in the vicinity of the cleavage sites. The enzyme did not display peptidyl dipeptidase activity with this substrate, and showed a specificity different from those reported for two other cysteine proteinases, papain and rat cathepsin L.

Cathepsin B is probably the most thoroughly characterized lysosomal cysteine proteinase, yet its peptide-bond specificity is unclear (Barrett & Kirschke, 1981). The oxidized B-chain of insulin has been used previously to assess the specificity of bovine spleen cathepsin B (Otto & Bhakadi, 1969; Keilova & Keil, 1969), but the results obtained by the two groups did not agree perfectly, and it is very likely that both enzyme preparations were contaminated to some extent with other proteinases, including cathepsin H (Barrett, 1977).

We decided to assess the action of a more thoroughly purified human liver cathepsin B on the oxidized B-chain of insulin. Cathepsin B acts as an endopeptidase on many substrates (Barrett, 1977), but displays a peptidyl dipeptidase activity against the C-terminus of glucagon (Aronson & Barrett, 1978) and rabbit muscle fructose biphosphate aldolase (Bond & Barrett, 1980). It was therefore considered to be of interest to determine how it would act on the oxidized B-chain of insulin.

Experimental

Materials

Insulin B-chain (oxidized) was purchased from Schwarz/Mann (Orangeburg, NY, U.S.A.), and cathepsin B was prepared from human or bovine liver by the methods of Barrett (1973) and Schwartz & Barrett (1980). Rat liver cathepsin B was a gift from Dr. Heidrun Kirschke (Physiologisch-Chemisches Institut der Martin-Luther-Universität, Halle, German Democratic Republic). Triethanolamine, Baker-Flex silica gel (1B2) plates and all solvents (spectrophotometric grade) for h.p.l.c. were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Dansyl chloride, fluorescamine and polyamide thin-layer plates were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Amino acid analysis reagents and buffers were obtained from Durrum (Sunnyvale, CA, U.S.A.). Dansyl-amino acid standards and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Incubation of insulin B-chain with cathepsin B

Insulin B-chain (10 mg) was dissolved in 0.5 ml of 0.2 M-triethanolamine (pH > 8.0), and the pH was adjusted to pH 6.0 by adding 0.5 M-acetic acid in 10 µl increments over a period of 40 min. EDTA and

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

‡ Present address: Department of Internal Medicine, University of Chicago, Chicago, IL 60637, U.S.A.

§ To whom correspondence should be addressed.

dithioerythritol (in a stock solution of 0.2M-triethanolamine/acetic acid, pH6.0) were added to render a final concentration of 1.1mM of each in 1.1 ml. The reaction was started by the addition of 100 μ l of cathepsin B (1mg/ml in distilled water). The reaction mixture was then incubated at 25°C for 20h on a continuous-rocking mixer. At the end of this incubation period, the reaction mixture was dried (1h) in a Savant Speed Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.).

Separation and analysis of degradation products

The dry reaction products were suspended in 1 ml of 1% (w/v) trifluoroacetic acid and centrifuged in a Beckman Microfuge B for 4min. The sediment consisted of undigested insulin B-chain (as shown by dansyl chloride and amino acid analyses), and was discarded. Samples of the clear supernatant fluid, containing 2.08mg of protein/ml, as determined by the Lowry *et al.* (1951) method, were subjected to h.p.l.c. with a Varian model 5000 liquid chromatograph (Varian Instruments, Palo Alto, CA, U.S.A.) on a Varian MCH-10 reverse-phase (C-18) column that had been equilibrated with 1% trifluoroacetic acid. A sample (100 μ l) of the supernatant fluid was applied to the column, and the column was flushed with 5ml of 1% trifluoroacetic acid. Retained material was then eluted from the column with a linear gradient (0–70%, v/v) of 75% (v/v) acetonitrile in 1% trifluoroacetic acid (solution B) at a flow rate of 1ml/min. The eluate was monitored at 215nm. Fractions (1 ml) of the eluate were collected in chromic acid-washed tubes. This procedure was repeated a number of times.

Fractions representing single peaks or groups of peaks of absorbance that were eluted in the same region of individual chromatographic separations were pooled and dried in a Savant Speed Vac

concentrator. These pooled samples were individually dissolved in a minimal volume of 50% (v/v) pyridine and subjected to t.l.c., in duplicate, on Baker-Flex silica gel (1B2) plates. The duplicate plates were run simultaneously in one dimension in butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.) for approx. 5h at 23°C. Peptide bands were located with fluoescamine staining (Schiltz *et al.*, 1977). Peptide bands scraped from one plate were used for N-terminal residue analysis by the dansyl chloride method with the use of polyamide t.l.c. (Gray, 1972). Peptide bands scraped off the second plate were hydrolysed with 6M-HCl in evacuated sealed tubes for 30h at 110°C, and then subjected to amino acid analysis in a Durrum MBF amino acid analyser with an *o*-phthalaldehyde detection system.

Comparative studies of the products of the degradation of insulin B-chain by rat, bovine and human liver cathepsins B were conducted as above except that analyses were performed with a Beckman model 332 liquid chromatograph (Beckman Instruments, Berkeley, CA, U.S.A.) and a Waters μ Bondapak C-18 reverse-phase column (Waters Associates, Milford, MA, U.S.A.).

Results

It was found that during the reaction period of 20h at pH6, a cathepsin B/insulin B-chain protein ratio of 1:100 resulted in the conversion of approx. 20% of the oxidized B-chain of insulin into trifluoroacetic acid-soluble peptides. H.p.l.c. of the trifluoroacetic acid-soluble material (Fig. 1) led to the identification of 16 peptides, designated I–XVI. The peaks recovered from the h.p.l.c. were subjected to t.l.c. Some peaks (e.g. IV, VIII, XI and XIV) were found to contain homogeneous peptides. For others, preparative t.l.c. was used to complete the separation

Fig. 1. H.p.l.c. of the soluble peptide fragments of the B-chain of insulin produced by human cathepsin B. A sample of (100 μ l) of the trifluoroacetic acid-soluble degradation products of insulin B-chain was subjected to h.p.l.c. as described in the Experimental section. This is a typical profile of the separated products. Solution B was 75% (v/v) acetonitrile in 1% (w/v) trifluoroacetic acid. The corresponding absorbance peaks from several h.p.l.c. separations were pooled, and the peptides associated with the numbered absorbance peaks were identified in subsequent analyses.

of a pair of peptides. Fig. 2 shows a typical t.l.c. plate after chromatography of fractions eluted at 55–60 ml from the h.p.l.c. column. Peak XVII was found to be intact insulin B-chain, and no other

Fig. 2. T.l.c. silica-gel chromatogram of the 55–60 ml eluate fraction from the h.p.l.c. separation of the degradation products of insulin B-chain

The group of peaks eluted in the 55–60 ml fraction of the h.p.l.c. separation were subjected to t.l.c. on silica-gel plates as described in the Experimental section. This sample (a) was applied adjacent to a sample of intact insulin B-chain (b); duplicate plates were chromatographed simultaneously. Region 1 of the Figure refers to the position of insulin B-chain, and region 2 refers to the two peptide bands (designated XII and XIII) as identified by fluorescamine. Region 3 is an area of the plate that was removed and subjected to amino acid analysis to serve as a monitor of 'background' amino acid contamination for each respective t.l.c. plate.

peptide peaks were eluted subsequently. The results of the peptide analyses are presented in Fig. 3. The designation of major and minor sites of cleavage (as indicated in line 5 of the Figure) was arrived at by consideration of the identity of the peptides and their relative amounts as determined by amino acid analysis.

The chromatographic profiles obtained as a result of degradation of insulin B-chain by human, rat or bovine cathepsin B were compared (Fig. 4); they appear to have a great deal of similarity. Of those peptide peaks detected (and labelled according to retention time) on the h.p.l.c. profile for the bovine enzyme, 98% were identified on the h.p.l.c. profile for the rat enzyme and 76% were identified on the h.p.l.c. profile for the human enzyme.

Discussion

The specificity of cathepsin B is peculiarly enigmatic. The bovine enzyme was discovered by use of a synthetic substrate for trypsin, benzoyl-arginine amide, and was considered to have a trypsin-like specificity (Greenbaum & Fruton, 1957). The first studies of the action of the enzyme on the oxidized insulin B-chain dispelled this idea, however: the arginyl and lysyl bonds were cleaved weakly, if at all. In fact, there was some resemblance to the broad specificity of papain (Fig. 3). Recent work on the primary sequence of cathepsin B has shown the *N*-terminal portion of the molecule, which contains the essential cysteine residue, to be extremely similar to that of papain (Takio *et al.*, 1980; Pohl *et al.*, 1982). The resemblance is such as to leave no doubt that the molecules are evolutionarily homologous, and this prompts closer comparison of

Fig. 3. Action of human liver cathepsin B on the oxidized B-chain of insulin

The sequences of the peptide fragments recovered from h.p.l.c. and t.l.c. analyses were determined by comparison with the known structure of the insulin B-chain. Designations of major (↑) and minor (†) sites of cleavage (line 5) were based on the amount of a particular peptide detected in h.p.l.c. analyses and the relative amounts of amino acids detected after amino acid analyses. The results are compared with the data of (1) Johansen & Ottesen (1968) for papain, (2) Kargel *et al.* (1980) for rat liver cathepsin L and (3) and (4) Otto (1971) and Keilova (1971) respectively for bovine spleen cathepsin B.

Fig. 4. *H.p.l.c. analyses of the degradation products of oxidized insulin B-chain after incubation with human, bovine or rat liver cathepsin B*

The oxidized B-chain of insulin was incubated with (b) human, (c) bovine or (d) rat liver cathepsin B, and the trifluoroacetic acid-soluble degradation products were subjected to h.p.l.c. as described in the Experimental section. The top panel (a) shows the position of intact oxidized B-chain of insulin in the elution profile.

the specificities of cathepsin B and papain. Berger & Schechter (1970) showed that the most important determinant of the specificity of papain is the residue P_2 (in their terminology), which should be hydrophobic and bulky, such as phenylalanine. Phenylalanine in P_2 also provides excellent substrates for cathepsin B, although the enzyme accepts arginine very well in P_2 also (Barrett & Kirschke, 1981), unlike papain.

Fruton (1975) has pointed out that papain has an extended specificity site, with important subsites on both sides of the catalytic site, and there is reason to think that the same is true of cathepsin B. One indication that residues on the carboxy side of the cleavage site (P_1' and P_2') may be significant for cathepsin B is the extremely rapid reaction with active-site-directed inhibitors that bind in the S_1' and S_2' subsites (Barrett *et al.*, 1982). The peptidyl-dipeptidase activity of cathepsin B against glucagon and muscle fructose biphosphate aldolase (Aronson & Barrett, 1978; Bond & Barrett, 1980) could

additionally imply an S_3' affinity for a carboxy group.

The specificity of human, rat and bovine cathepsins B deduced from our data (Figs. 3 and 4) differs in several major cleavage points from those reported by Otto & Bhakadi (1969) and Keilova & Keil (1969) for the specificity of bovine cathepsin B. One factor contributing to the differences may be the more complete purification of cathepsin B now possible. Otto (1971) commented on the difficulty of removing the leucine naphthylamidase activity (now known to be due to cathepsin H) from cathepsin B preparations, and there is no doubt that the enzyme used earlier in his study of the degradation of insulin B-chain will have had some such contamination. The same may well be true of the preparation used by Keilova (1971). Thus the splits at $\text{His}_{(5)}\text{-Leu}_{(6)}$, $\text{His}_{(10)}\text{-Leu}_{(11)}$, $\text{Ala}_{(14)}\text{-Leu}_{(15)}$ and $\text{Leu}_{(17)}\text{-Val}_{(18)}$ reported by Otto (1971) and Keilova (1971) may have been due to cathepsin H, but we have no explanation for the apparent absence of cleavages at

Gln₍₄₎-His₍₅₎ and Ser₍₉₎-His₍₁₀₎ in the earlier work. It is conceivable that the species source of cathepsin B may have been responsible for the differences in peptide-bond cleavage. Our initial results (Fig. 4), however, indicate that there are no marked differences in the products produced by cathepsin B from different species.

Our data reveal no simple pattern in the specificity of cathepsin B. The P₂ residues for the major cleavage sites are not consistently hydrophobic (Asn, Gly, Val, Leu, Arg), but it is possibly significant that the P₂' residues in this sample tend to be hydrophobic (Leu, Leu, Leu, Val, Phe). We found a smaller number of significant cleavage points than did the earlier workers, again perhaps because of the higher purity of the enzyme preparations now available. We conclude that cathepsin B shows a distinct specificity, different from that reported for papain, but one that is not yet adequately explicable in terms of the requirements of individual specificity sites.

This work was supported by National Institutes of Health Grant AM 19691. J. S. B. is the recipient of a Research Career Development Award from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

References

- Aronson, N. N. & Barrett, A. J. (1978) *Biochem. J.* **171**, 759–765
- Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822
- Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 181–208, Elsevier/North-Holland Biomedical Press, Amsterdam
- Barrett, A. J. & Kirschke, H. (1981) *Methods Enzymol.* **80**, 535–561
- Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. & Hanada, K. (1982) *Biochem. J.* **201**, 189–198
- Berger, A. & Schechter, I. (1970) *Philos. Trans. R. Soc. London. Ser. B* **257**, 249–264
- Bond, J. S. & Barrett, A. J. (1980) *Biochem. J.* **189**, 17–25
- Fruton, J. S. (1975) in *Proteases in Biological Control* (Reich, E., Rifkin, D. & Shaw, E., eds.), pp. 33–50, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Greenbaum, L. M. & Fruton, J. S. (1957) *J. Biol. Chem.* **226**, 173–180
- Johansen, J. T. & Ottesen, M. (1968) *C. R. Trav. Lab. Carlsberg* **36**, 265–272
- Kargel, H. J., Dettmer, R., Etzold, G., Kirschke, H., Bohley, P. & Langner, J. (1980) *FEBS Lett.* **114**, 257–260
- Keilova, H. (1971) in *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 45–65, North-Holland, Amsterdam
- Keilova, H. & Keil, B. (1969) *FEBS Lett.* **4**, 295–298
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Otto, K. (1971) in *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 1–28, North-Holland, Amsterdam
- Otto, K. & Bhakadi, S. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1577–1588
- Pohl, J., Baudys, M., Tomasek, V. & Kostka, V. (1982) *FEBS Lett.* **142**, 23–26
- Schiltz, E., Schnackers, K. D. & Gracy, R. W. (1977) *Anal. Biochem.* **79**, 33–41
- Schwartz, W. N. & Barrett, A. J. (1980) *Biochem. J.* **191**, 487–497
- Takio, K., Towatari, T., Katunuma, N. & Titani, K. (1980) *Biochem. Biophys. Res. Commun.* **97**, 340–346