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Crystallization of Recombinant Rat Cathepsin B*

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A glycosylation-minus mutant of rat cathepsin B expressed in yeast has been purified and crystallized. X-ray diffraction data have been collected and molecular replacement for solving the structure is in progress. The space group for the recombinant rat cathepsin B was determined to be $P2_1$ with unit cell dimensions $a = 62.2$ Å, $b = 90.19$ Å, $c = 47.07$ Å, and $\beta = 97.43°$. A unit cell contains 4 molecules and 2 molecules per asymmetric unit.

Cathepsin B is a lysosomal cysteine protease (EC 3.4.22.1) which has been purified from a number of mammalian and avian sources (1, 2). It is synthesized as an inactive precursor which is activated by limited proteolysis resulting in a mature enzyme consisting of two polypeptide chains of M_r 24,000 and 5,000. Nucleotide sequence analysis of cDNA clones for both the rat and human cathepsin B genes predicts that the primary structure of preprocathepsin B contains 339 amino acids consisting of a 17-residue prepeptide, a 62-residue profragment, the 254-residue mature enzyme, and 6-residue extension at the COOH terminus (3, 4). In mammalian tissues processing events include removal of the profragment as well as the 6-residue COOH-terminal extension and a dipeptide at positions 48 and 49 of the mature enzyme sequence, generating the two-chain form of the enzyme. Usually some of the single-chain mature enzyme is also present in purified preparations. Cathepsin B is glycosylated at Asn-113 (single-chain numbering) which results in molecular mass estimation on the basis of SDS-PAGE[1] of 29,000 Da as compared to a theoretical calculation of 27,500 Da based on the primary sequence.

Active rat cathepsin B has been expressed in yeast by Mort.[2] The secreted enzyme was shown to be heterogeneously glycosylated on the basis of SDS-PAGE and gel exclusion chromatography.[3] For this reason a mutant was constructed (Ser115Ala) to eliminate glycosylation in yeast.[2]

Because of the implication of cathepsin B in a number of disease states such as muscular dystrophy, arthritis and inflammation, and myocardial infarcts, and interest in developing inhibitors for possible therapeutics, (7–10), we undertook to crystallize cathepsin B for the determination of its structure by x-ray crystallography. To date there has been only one report of crystallization of cathepsin B (11), but we assume the spindle-shaped crystals were not suitable for x-ray crystallography since there has been no report on data collection. Their crystals were produced by simple addition of ammonium sulfate to concentrated purified enzyme which was then stored in the cold until crystals appeared. Here we report the first production of crystals suitable for x-ray crystallography of recombinant rat cathepsin B and give preliminary results on the diffraction studies.

EXPERIMENTAL PROCEDURES

Cloning and Expression—The cDNA for rat procathepsin B (3) was expressed in *Saccharomyces cerevisiae* as an α-factor fusion.[2] A mutant of rat cathepsin B (rCat B G−) was constructed by site-directed mutagenesis to eliminate *N*-linked glycosylation.[2] The serine residue at position 115 (numbering for single chain of mature cathepsin B) was converted to an alanine, thus removing the consensus sequence for *N*-linked oligosaccharide substitution.

Purification of rCat B Expressed in Yeast—The rCat B G− mutant was maintained in *S. cerevisiae* by fermentation in synthetic medium without casamino acids. Pre-cultures were used to innoculate 1–2 liters of the SD medium with casamino acids (12) in 4-liter shake flasks with baffles, and cultures were grown at 30 °C and 200 rpm in a New Brunswick shaking incubator. Cell growth and cathepsin activity in the culture filtrate were monitored spectrophotometrically. When enzymatic activity reached a maximum in the culture filtrate, cells were separated from the culture medium by centrifugation. The supernatant was concentrated 30-fold using a Millipore Pellicon concentrator fitted with a 10,000-Da cutoff Millipore PTGC membrane. The concentrate was clarified by centrifugation and further concentration in an Amicon concentrator fitted with a YM-5 filter.

The concentrate was dialyzed against 20 mM sodium phosphate, 1 mM EDTA, 0.1% Brij-35, pH 6.0, and then applied to a DEAE-cellulose column (DE52, Whatman) preequilibrated with 20 mM phosphate, 1 mM EDTA, 0.1% Brij-35, pH 6.0. The bound enzyme was eluted stepwise with additions of up to 300 mM NaCl in the starting buffer. The eluted enzyme activity was pooled and concentrated in an Amicon ultrafiltration apparatus to a concentration of ~2 mg/ml. The concentrated enzyme was then applied to a column of Sephadex G-75 (Pharmacia LKB Biotechnology Inc.) equilibrated in 50 mM phosphate, 1 mM EDTA, 0.001% Brij-35, pH 6.0. Fractions with similar specific activity were pooled and concentrated. Purity was confirmed by SDS-PAGE using a Pharmacia Phast-Gel system (Fig. 1, *lane 2*). The molecular mass of the recombinant rCat B G− mutant was calculated to be approximately 29,000 Da on the basis of SDS-PAGE and therefore was present only as the single-chain form in contrast to rat liver and human liver cathepsin B (Fig. 1, lanes 3 and *4*). This molecular mass is larger than the theoretical value of 27,700 Da for native nonglycosylated rCat B because the recombinant enzyme has a 6-amino acid residue extension at the NH_2 terminus due to incorrect processing in yeast.[2] We believe that the COOH terminus may also be longer by 6 residues compared to the native rat enzyme, because it is unlikely that the yeast has the specific processing enzymes found in mammalian systems. These extensions are also responsible for lowering the pI of the recombinant enzyme (data not shown) due to additional acidic residues in the extensions. Kinetic characterization[4] has shown that the recombinant enzyme is virtually identical as far as function is concerned to the native rat liver cathepsin B.

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[1] The abbreviation used is: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[2] J. S. Mort, manuscript in preparation.

[3] S. Hasnain and J. S. Mort, unpublished results.

[4] S. Hasnain, unpublished results.

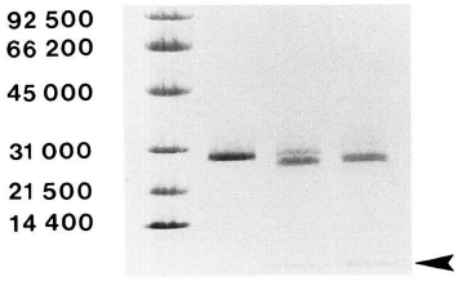

FIG. 1. **SDS-polyacrylamide gel electrophoresis of purified cathepsins on a Phast 8–25% gradient gel.** *Lane 1*, molecular mass markers: phosphorylase *b*, 92,500 Da; bovine serum albumin, 66,200 Da; ovalbumin, 45,000 Da; carbonic anhydrase, 31,000 Da; soybean trypsin inhibitor, 21,500 Da; lysozyme, 14,400 Da. *Lane 2*, recombinant rat cathepsin B glycosylation-minus mutant expressed in yeast; *lane 3*, native rat liver cathepsin B; *lane 4*, human liver cathepsin B. The *arrowhead* indicates the position of the small chain of the native rat liver and human liver enzymes (*lanes 3* and *4*, respectively).

Enzyme Assays—Cathepsin B activity was determined by a continuous spectrophotometric assay using *N*-benzyloxycarbonyl-L-arginyl-L-arginine-*p*-nitroanilide, and nitroaniline release was monitored at 410 nm at room temperature. The reaction buffer contained 100 mM phosphate, 1 mM EDTA, 10 mM dithiothreitol, pH 6.0. The enzyme was activated in reaction buffer for ~5 min and the reaction was started by addition of substrate to a final concentration of 0.8 mM. The reaction was followed and activity was determined from the linear portion of the optical density profile.

Active Enzyme Determination—The percent active enzyme was determined by free thiol titration using E-64 ([L-3-*trans*-carboxyoxiran-2-carbonyl]-L-leucyl-agmatin) according to the method of Barrett and Kirschke (1). Active enzyme concentration was usually 75% or more of the total purified enzyme concentration.

RESULTS AND DISCUSSION

Crystallization and X-ray Data Collection—The protein concentration for crystallization of the recombinant r Cat B G– mutant was 8.0 mg/ml. Crystals were grown by the hanging drop method of vapor diffusion. The reservoir contained 0.8 M lithium sulfate buffered to pH 4.4 with 0.1 M sodium citrate. Droplets were made up of 5 μl of protein solution (8.0 mg/ml) and 5 μl of the reservoir solution. Crystals appeared after 1 week and they grew to a reasonable size (0.5 × 0.35 × 0.2 mm) for data collection after 3 weeks.

Preliminary x-ray data indicating the symmetry and approximate cell dimensions were collected with a precession camera and CuKα radiation (40 kV, 200 mA) from a rotating anode x-ray generator (Rigaku RU-200 VH) at 22 °C. The space group was determined as $P2_1$ with a = 62.21 Å, b = 90.19 Å, c = 47.07 Å, and β = 97.43°. The cell volume is 261,860 Å^3 which corresponds to a volume/mass ratio of 2.3 Å^3/Da, with 2 molecules in an asymmetric unit and 4 molecules/unit cell. A complete set of data to 2.4 Å has been collected with an area detector (San Diego Multiwire Systems). The R_{sym} value:

$$\frac{\sum_{ij}\sum[\bar{I}_i - I_j]}{\sum_j I_j}$$

over j observations of i reflections, is 6.9% with an average redundancy of 4.2. Molecular replacement for solving the structure is under way while attempts to obtain larger crystals are still being made.

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