Construction of a Modified Penicillin-Binding Protein 2a from Methicillin-Resistant Staphylococcus aureus and Purification by Immobilized Metal Affinity Chromatography

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The mecA-27r gene, which encodes PBP2a-27r, was modified by site-specific mutagenesis, resulting in replacement of the N-terminal membrane anchor with a short chelating peptide (CP-PBP2a-27r). CP-PBP2a-27r retained the same binding affinity for β-lactam antibiotics as the wild-type enzyme. Approximately 95% pure CP-PBP2a-27r was recovered in a single step by use of chelating-peptide-immobilized metal ion affinity chromatography.

Methicillin-resistant staphylococci cause serious nosocomial infections. These infections represent a leading cause of morbidity and mortality in hospitals and chronic care facilities (2, 6). Resistance to methicillin in staphylococci has been associated with the production of a membrane-bound, high-molecular-weight penicillin-binding protein (PBP2a) which exhibits weak affinity for penicillins and cephalosporins (1, 8). Presumably, PBP2a can substitute for the transpeptidase activity of other penicillin-binding proteins and allow survival of the organism in the presence of otherwise lethal concentrations of β-lactam antibiotics (5). The mecA gene, which encodes PBP2a, has been cloned, sequenced, and analyzed by in vitro mutagenesis (7, 9, 11, 12). PBP2a is composed of three domains: an N-terminal membrane-spanning region which anchors PBP2a to the external surface of the cell membrane, a putative transglycosylase domain, and a transpeptidase domain containing the target of β-lactam antibiotics. Membrane-bound proteins are generally difficult to purify and crystallize. In a structure-based drug design approach for the development of an inhibitor for PBP2a, the mecA-27r gene from the methicillin-resistant Staphylococcus aureus strain 27r was modified by removing the region that encodes the membrane-spanning region. Expression of the modified mecA-27r gene in Escherichia coli resulted in production of a fully active water-soluble form of PBP2a-27r (12). At that point, a facile purification process was designed for recovery of PBP2a that would be suitable for further studies. Kasher and colleagues have described a single-step purification of recombinant human papillomavirus type 16 E7 oncoprotein by use of chelating-peptide-immobilized metal ion affinity chromatography (CP-IMAC) (3). Here we report the construction of another modified form of PBP2a-27r and the application of CP-IMAC for purification of PBP2a-27r.

In order to apply the CP-IMAC process to the purification of PBP2a-27r, the mecA-27r gene was modified by in vitro mutagenesis (4). An NcoI restriction site followed by a sequence that encodes a short chelating peptide (MGHWHHH) was inserted by site-specific mutagenesis just upstream of, and in frame with, the triplet which codes for amino acid residue 23 of PBP2a-27r. The synthetic oligodeoxynucleotide used as the mutagenic primer is shown in Fig. 1A. Alterations induced in PBP2a-27r are illustrated in Fig. 1B. The template for mutagenesis was a portion of the mecA-27r gene cloned into M13. This clone carried the 5′ region of the mecA-27r gene (12). After mutagenesis, the modified 5′ end of the mecA-27r gene was reattached to the remainder of mecA-27r in an E. coli expression vector as described previously (12). The resulting plasmid, pEWSA31, (Fig. 1C), contained the modified mecA-27r gene encoding an altered version of PBP2a-27r in which the transmembrane domain was removed and replaced with a short chelating peptide (CP-PBP2a-27r). Expression of the modified mecA-27r gene in plasmid pEWSA31 was under the control of the heat-inducible lambda P1 promoter.

Modifications made to the protein may have affected its ability to bind penicillin. Therefore, it was necessary to examine the interaction of CP-PBP2a-27r with β-lactams. Crude cell extracts from E. coli cells transformed with pEWSA31 were used to assess the penicillin-binding capacity of CP-PBP2a-27r. When tested for binding activity in a competition assay using cefamandole in competition with β-lactamase V, the 50% inhibitory concentration for CP-PBP2a-27r was estimated to be between 90 and 180 μg of cefamandole per ml (Fig. 2). The wild-type form of this enzyme from staphylococcal membranes also exhibited a 50% inhibitory concentration of between 90 and 180 μg of cefamandole per ml under similar conditions (12). Similar results were obtained with other β-lactam compounds (data not shown). The removal of the transmembrane domain and its replacement with the short chelating peptide appeared not to affect the β-lactam-binding efficiency of the enzyme.

CP-PBP2a-27r was produced by introducing plasmid pEWSA31 into E. coli DH5α and exposing these cells to conditions that induced expression of the modified mecA-27r gene. A sample from an overnight broth culture of E. coli DH5α containing plasmid pEWSA31 was streaked onto TY (Bacto-Tryptone, 10 g/liter; Bacto-yeast extract, 5 g/liter; Bacto-Agar, 15 g/liter) agar plates containing 10 μg of tetracycline per ml. The inoculated plates were incubated for 4 h at 30°C. Expression of CP-PBP2a-27r was induced by increasing the temperature of incubation to 41°C for an additional 4 h. Under these conditions, the bulk of CP-PBP2a-27r was found in inclusion bodies (granules). Alternatively, a broth culture could be used for expression. To isolate granules containing CP-PBP2a-27r, 1 g of cells (wet weight) was enzymatically
digested by suspension in 10 ml of a solution containing 4 mg of lysozyme, 5 mM EDTA, and 50 mM Tris (pH 8.0). The cell suspension was incubated at room temperature for 15 min or until the solution became viscous. The lysozyme-treated cell suspension was cooled on ice. The chilled suspension was sonicated three times with a sonic dismembrator to lyse the cells (30-s sonic bursts with a 1-min interval between bursts, with cell suspension on ice between bursts). The cell lysate was added to 30 ml of 50 mM Tris (pH 8.0) containing 2 g of Whatman DEAE-cellulose, mixed, and filtered through a Buchner funnel with Whatman no. 1 filter paper. A weak vacuum was applied, and the DEAE trapped on the filter was washed with Tris buffer until the filtrate cleared. The filtrate containing the granules was centrifuged at 4,300 × g for 20 min. The pellet was washed first in 20 ml of 0.1% Triton–10 mM EDTA, then in 20 ml of 1 mM KCl, and finally in 20 ml of distilled water. Granules harvested by centrifugation (12,000 × g for 20 min) from the final wash were resuspended in water and stored at –20°C.

In order to proceed with protein purification, it was necessary to release the proteins trapped in the granules. Granules containing CP-PBP2a-27r were solubilized in sulfitolysis reagent (7.5 M urea, 0.5 M Tris-HCl, 100 mM Na2SO3, 10 mM Na2S2O5, pH 8.2) and filtered through a 0.45-μm-pore-size filter before application to the Zn(II) IMAC purification column. Lane 1 of the inset in Fig. 3 illustrates the proteins released from the granules.

The CP-IMAC purification procedure used to purify CP-PBP2a-27r was modified from the method described by Kasher and coworkers (3). Briefly, an HR 10/10 Pharmacia column was poured with Pharmacia Fast-Flow Chelating Gel, connected to a fast protein liquid chromatography LCCS00 fraction collection system (Pharmacia Biotech, Piscataway, N.J.), and washed with 5 ml of distilled water. The metal ion [Zn(II)] was loaded onto the column by applying 6 ml of a 50 mM ZnCl2 solution to the column. The metal-loaded column was washed with 5 ml of distilled water and equilibrated with buffer A (50 mM NaH2PO4, 0.5 M NaCl, 7 M urea, pH 8.0). A 2-ml sample of the sulfitolysis solution containing the solubilized proteins from granules was loaded onto the column. The column was washed with buffer A at 0.25 ml/min until the A280 of the eluate was negligible, generally about 30 min. The bound material was then eluted by introducing a displacing ligand. Buffer B, used to generate the imidazole gradient, consisted of 0.5 M imidazole, 50 mM NaH2PO4, 0.5 M NaCl, and 7 M urea (pH 8.0). A gradient of 0 to 60% buffer B was applied to the column over 90 min to elute CP-PBP2a-27r. The majority of the protein was eluted in the first peak, as illustrated by the A280 tracing in Fig. 3. CP-PBP2a-27r was eluted with an ascending gradient of imidazole as a single peak of protein.
FIG. 3. Zn(II) CP-IMAC column purification of PBP2a-27r. The eluant obtained from the column was fractionated and analyzed for the presence of an enriched protein band. The solid line represents the \( A_{280} \) of the eluant. The dashed line represents the ascending imidazole gradient from 0 to 500 mM. The numbers at the bottom indicate the fraction numbers collected. The photograph of the Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel superimposed above the second peak illustrates sample applied to the column (lane 1), fraction 20 (lane 2), fraction 21 (lane 3), and fraction 22 (lane 4). A penicillin-binding assay was used to verify that the protein band indicated by arrow was CP-PBP2a-27r.

centered on fraction 21 (Fig. 3). In this example, a small quantity of approximately 95% pure CP-PBP2a-27r was obtained in a single pass over the Zn(II) IMAC column. Penicillin-binding assays once again confirmed that this protein retained its penicillin-binding activity.

Purification by use of CP-IMAC technology is an attractive alternative to conventional purification schemes. Many proteins containing chelating peptides have been purified to homogeneity in a single step by CP-IMAC (10). In this example, we purified a protein with a molecular mass of roughly 74 kDa (12), illustrating that the small chelating peptide used efficiently immobilized a rather large protein. CP-IMAC technology provided a viable alternative for rapid purification of this medically important protein.

REFERENCES