Penicillin-Binding Proteins in the Soluble Fraction of *Caulobacter crescentus*

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Four penicillin-binding proteins (PBPs) were observed in the soluble fraction of *Caulobacter crescentus* CB13, and designated PBP S1 (mol. wt 60 000), PBP S2 (55 000), PBP S3 (45 000) and PBP S4 (40 000). *Caulobacter crescentus* CB15, an independent isolate, possessed similar soluble PBPs, but not PBP S2. These soluble PBPs could be observed even when a cell suspension was directly reacted with [¹⁴C]penicillin G. Two of the soluble PBPs, S1 and S2, released half their bound [¹⁴C]penicillin G during a 10 min incubation, indicating that these PBPs have penicillinase-like activity. PBP S2 was very thermolabile and lost its penicillin-binding activity after incubation at 40 °C for 10 min. Mecillinam did not show selective binding to any of the *Caulobacter* soluble PBPs or to PBPs in the cell envelope.

INTRODUCTION

The Gram-negative stalked bacterium *Caulobacter* is characterized by the presence of two identical chromosomes in one cell (Iba & Okada, 1980), by a unique cell division pathway where functionally and morphologically different daughter cells are produced, and by a sequence of events which leads to the morphogenesis of surface structures at the poles of the cell at a defined time in the growth cycle (Poindexter, 1964, 1981; Shapiro, 1976). Recent studies have revealed that penicillin-binding proteins (PBPs) of bacteria play essential roles in cell wall formation, cell division and cell morphogenesis (Spratt, 1975, 1977*b*; Tamaki *et al.*, 1977; Iwaya *et al.*, 1978; Suzuki *et al.*, 1978). *Caulobacter* species possess PBPs in their cell envelope which are markedly different, in molecular weight and properties, from those of other bacteria.

It was reported previously (Koyasu *et al.*, 1980*a*, 1981) that several *Caulobacter* strains possessed at least five major PBPs, namely PBP1A (mol. wt 132 000), PBP1Bs (98 000), PBP2 (77 000), PBP3 (64 000) and PBP4 (50 000). One of the strains, *C. crescentus* CB13, lacked PBP4. However, no low molecular weight PBPs equivalent to PBP5 and PBP6 of *Escherichia coli* or other genera were detected (Shepherd *et al.*, 1977; Chase *et al.*, 1978; Curtis *et al.*, 1979; Noguchi *et al.*, 1979; Ohya *et al.*, 1979; Coyette *et al.*, 1980). In addition, none of the *Caulobacter* envelope PBPs showed penicillinase activity. In many other bacteria, PBPs of low molecular weight possess penicillinase activity (Shepherd *et al.*, 1977; Spratt, 1977*a*; Chase *et al.*, 1978; Noguchi *et al.*, 1979; Ohya *et al.*, 1979). *Caulobacter* also apparently lacks the mecillinam target equivalent to PBP2 of *E. coli* (Spratt & Pardee, 1975). Finally, PBP4 of *Caulobacter* was located in the outer membrane fraction, in contrast to other bacterial PBPs which are located only in the inner membrane (Spratt, 1977*a*; Noguchi *et al.*, 1979; Koyasu *et al.*, 1980*a*).

In addition to the well-characterized envelope PBPs, recent studies have indicated the presence of soluble PBPs in *Streptomyces* (Leyh-Bouille *et al.*, 1977; Ogawara & Horikawa, 1980), *E. coli* (Koyasu *et al.*, 1980*b*) and *Streptococcus* (Coyette *et al.*, 1980). The functions

of soluble PBPs are not clear at the moment, but it was of interest to investigate possible PBPs in a stalked bacterium and to study any relationship to morphogenesis in this organism. We describe in this paper four possible soluble PBPs in *C. crescentus*.

METHODS

Bacterial strains and growth conditions. *Caulobacter crescentus* wild-type strains CB13 and CB15 were used. The bacteria were grown in polypeptone/yeast extract (PYE) nutrient broth at 30 °C.

Fractionation of cell components. Bacteria were harvested from 2-litre cultures in the late-exponential phase of growth, washed with 10 mM-sodium phosphate buffer, pH 7.0, suspended in 10 ml of the same buffer and sonicated by a Branson Sonifier at setting 5 for 10 min at 0 °C. Unbroken cells were removed by low-speed centrifugation. The homogenates were centrifuged at 100 000 *g* for 30 min at 4 °C. The sediments (envelopes) were washed with 10 mM-sodium phosphate buffer, pH 7.0, and suspended in 1 ml of the same buffer. The supernatant fluid was further centrifuged at 100 000 *g* for 3.5 h at 4 °C. The upper and lower portions of the supernatant were recovered separately as S100 and S100' fractions, respectively. The ribosomal sediments (ribosomes containing remaining small vesicles of cell envelopes) were suspended in 1 ml 10 mM-sodium phosphate buffer, pH 7.0. Protein recovery of each fraction was determined by the Lowry method with bovine serum albumin as the reference protein. All samples were stored at -70 °C.

Binding of [¹⁴C]penicillin G and release of bound [¹⁴C]penicillin G. Penicillin-binding proteins were assayed as described previously (Koyasu *et al.*, 1980*a*, 1981). Briefly, samples, 30 µl in volume or 240 µg protein-equivalent, were incubated with 3 µl [¹⁴C]penicillin G at various concentrations at 30 °C for 10 min, mixed with 3 µl of a solution containing 3.4% (w/v) Sarkosyl and 6% (w/v) non-radioactive penicillin G and further incubated at 20 °C for at least 20 min. The mixture was centrifuged at 15 000 *g* for 15 min. To 30 µl of the supernatant were added 15 µl gel sample buffer [0.2 M-Tris/HCl, pH 6.8, 3% (w/v) sodium dodecyl sulphate (SDS), 30% (w/v) glycerol, 0.002% (w/v) bromophenol blue] and 5 µl β-mercaptoethanol. The mixture was heated at 90 °C for 2 min before electrophoresis.

The rate of release of bound [¹⁴C]penicillin G from PBPs was measured as described previously (Spratt, 1977*a*; Koyasu *et al.*, 1981). Samples (30 µl) were first incubated with [¹⁴C]penicillin G (200 µg ml⁻¹) at 30 °C for 10 min, and then mixed with 3 µl non-radioactive penicillin G (180 mg ml⁻¹) and further incubated at 30 °C. After different times, 2 µl portions of 5% Sarkosyl were added and the remaining radioactivity of bound [¹⁴C]penicillin G was assayed by electrophoresis and fluorography.

Binding of [¹⁴C]penicillin G to intact cells. Bacteria were grown in PYE medium, harvested in the late-exponential phase of growth and suspended in 10 mM-sodium phosphate buffer, pH 7.0 (about 10¹¹ cells ml⁻¹ or 100 mg wet weight ml⁻¹). A 30 µl portion of the cell suspension was incubated with 3 µl of various concentrations of [¹⁴C]penicillin G at 30 °C for 10 min, mixed with 3 µl of a solution containing 3.4% Sarkosyl and 6% non-radioactive penicillin G and incubated at 20 °C for at least 20 min. The mixture was then centrifuged at 15 000 *g* for 15 min. To 30 µl of the supernatant were added 15 µl gel sample buffer and 5 µl β-mercaptoethanol. The mixture was heated at 90 °C for 2 min before electrophoresis. The above concentration of Sarkosyl was enough to solubilize all PBPs. The use of higher concentrations of Sarkosyl to solubilize additional envelope components caused severe disturbance of the subsequent electrophoretic analysis.

Affinities of β-lactam antibiotics for PBPs. The pre-addition method (Spratt, 1977*a*; Koyasu *et al.*, 1981) was used. Samples, 27 µl in volume or 220 µg protein-equivalent, were incubated with 3 µl of a β-lactam antibiotic at various concentrations at 30 °C for 10 min, then mixed with 3 µl [¹⁴C]penicillin G (final concentration 100 µg ml⁻¹) and further incubated at 30 °C for 10 min. The binding was terminated by the addition of 3 µl of a solution containing 3.4% Sarkosyl and 6% non-radioactive penicillin G.

Slab gel electrophoresis and fluorography. The gel was 2 mm thick. Two different SDS-polyacrylamide gel systems were used. The running gel was 7.5% (w/v) acrylamide, 0.1% (w/v) (system 1) or 0.05% (system 2) *N,N'*-methylenebisacrylamide, 0.1% SDS in 0.375 M-Tris/HCl, pH 8.9; the stacking gel was 3.75% acrylamide, 0.05% *N,N'*-methylenebisacrylamide, 0.1% SDS in 0.25 M-Tris/HCl, pH 6.8. The running buffer was 0.05 M-Tris/0.384 M-glycine/0.1% SDS. Electrophoresis was carried out at 25 mA for 6 h at room temperature.

Fluorography was carried out as described by Bonner & Laskey (1974) and Laskey & Mills (1975). The gel was impregnated with 2,5-diphenyloxazole, dried on a filter paper and exposed to a pre-fogged Kodak X Omat R X-ray film at -80 °C for at least 3 weeks. For quantitative analysis, the film was cut into strips and scanned by a Gilford gel scanner. The peak area was measured and corrections were made for differences in the width of gel slots when necessary.

Chemicals. [¹⁴C]Penicillin G (50 to 60 Ci mol⁻¹; 1.8 to 2.2 TBq mol⁻¹) was purchased from Amersham. Apalcillin, cephaloridine, penicillin G and mecillinam were obtained from Sumitomo Chemical Co., Osaka, Takeda Pharmaceutical Industries Co., Osaka, Meiji Seika Co., Tokyo and Leo Pharmaceutical Products, Denmark, respectively.

RESULTS

Presence of PBPs in the soluble fraction. In order to test whether PBPs exist in the soluble fraction, *C. crescentus* CB13 cell components were fractionated as described in Methods. The protein content of each fraction from a 2-litre culture was as follows: homogenate, 320 mg; supernatant after centrifuging at 100 000 g for 30 min, 200 mg; cell envelopes, 47 mg; S100 fraction, 32 mg; S100' fraction, 71 mg; ribosomal fraction, 71 mg. PBPs were assayed in each fraction by using gel system 2, by which clear separation of high molecular weight PBP1Bs may be achieved (Fig. 1), or by gel system 1, by which clear separation of low molecular weight PBPs may be achieved (Fig. 2).

In the soluble S100 fraction (Fig. 1c, Fig. 2c), at least four new PBPs were clearly detected in the low molecular weight regions of the gel. The cell envelopes (Fig. 1f) contained PBPs of high molecular weight as reported previously (Koyasu *et al.*, 1980a, 1981). These soluble PBPs were designated PBP S1, PBP S2, PBP S3 and PBP S4, having apparent molecular weights of 60 000, 55 000, 45 000 and 40 000, respectively. A minor band of molecular weight 42 000 and a few additional bands of low molecular weight were sometimes detected (Fig. 1c, d; Fig. 2c). Strong sonication probably produces minute membrane vesicles which may contaminate the soluble fraction. When cells were sonicated only briefly, the above PBPs were still observed exclusively in the soluble fraction (S100 and S100' fractions).

These PBPs were observed even when phenylmethylsulphonylfluoride, a serine protease inhibitor, was present during preparation of the S100 fraction. When the envelopes were incubated at 30 °C prior to the PBP assay, the soluble PBPs did not appear. When the homogenates were incubated at 30 °C prior to the PBP assay, the amounts of the soluble PBPs did not change.

For comparative purposes, soluble S100 fraction of *C. crescentus* CB15 was prepared and reacted with [¹⁴C]penicillin G. As shown in Fig. 2, the soluble PBPs of the two strains of *Caulobacter* were quite similar: strain CB15 possessed PBP S1, PBP S3, PBP S4 and an additional band of molecular weight of 35 000, but did not possess PBP S2.

Binding of [¹⁴C]penicillin G to PBPs in intact cells and in the soluble fraction. A suspension of intact bacteria was reacted with [¹⁴C]penicillin G and, as shown in Fig. 2, all PBPs that were detected previously in the soluble fraction were also detected in the intact cell.

In order to examine the binding kinetics of [¹⁴C]penicillin G to PBPs in the soluble fraction and in the intact cell, cell suspensions or the soluble S100 fraction of *C. crescentus* CB13 was incubated with [¹⁴C]penicillin G at various concentrations. As shown in Fig. 3, the binding kinetics of [¹⁴C]penicillin G to all PBPs were very similar using both methods. [¹⁴C]Penicillin G showed a high affinity for PBP1A and PBP1Bs. These PBPs were nearly saturated with this antibiotic at 1 µg ml⁻¹. PBP2 and PBP3 required a higher concentration of the antibiotic for saturation. Among the soluble PBPs, [¹⁴C]penicillin G showed the highest affinity for PBP S3 and the next highest for PBP S2. PBP S1 and PBP S4 did not seem to be saturated with [¹⁴C]penicillin G even at 100 µg ml⁻¹. [¹⁴C]Penicillin G was shown to have a lower affinity for some PBPs when added to the intact cells, indicating some effect of the outer membrane permeability barrier to the antibiotic.

Release of bound [¹⁴C]penicillin G from soluble PBPs. Some bacterial PBPs, particularly those of low molecular weight, possess penicillinase-like activity and release bound [¹⁴C]penicillin G rapidly during incubation (Shepherd *et al.*, 1977; Spratt, 1977a; Chase *et al.*, 1978; Noguchi *et al.*, 1979; Ohya *et al.*, 1979). PBPs of envelope origin in *C. crescentus* CB13 and CB15 did not have such an activity (Koyasu *et al.*, 1981), and it was of interest to test for penicillinase-like activity of the soluble PBPs.

We examined the release of bound [¹⁴C]penicillin G from soluble PBPs as described in Methods. PBP S1 and PBP S2 released bound [¹⁴C]penicillin G rapidly. Half the amount of bound [¹⁴C]penicillin G was released from these PBPs during 10 min incubation, while other soluble PBPs did not release bound [¹⁴C]penicillin G after 1 h incubation.

Fig. 1. Penicillin-binding proteins in each fraction of the cell. Samples were incubated with [^{14}C]penicillin G ($100\text{ }\mu\text{g ml}^{-1}$) at $30\text{ }^{\circ}\text{C}$ for 10 min, mixed with Sarkosyl and non-radioactive penicillin G, and subjected to SDS—polyacrylamide gel electrophoresis (system 2) and subsequent fluorography as described in Methods. The numbers on the left side are the designations of the PBPs. (a) Homogenate; (b) supernatant after centrifuging at $100\,000\text{ g}$ for 30 min; (c) S100 fraction; (d) S100' fraction; (e) ribosomal fraction; (f) cell envelopes.

Fig. 2. [^{14}C]Penicillin G binding to PBPs in the soluble fraction and in the intact cell. Samples were reacted with [^{14}C]penicillin G as described in Methods. The concentrations of [^{14}C]penicillin G were $100\text{ }\mu\text{g ml}^{-1}$ (a, b, c, d, f) or $300\text{ }\mu\text{g ml}^{-1}$ (e). Gel system 1 was used. The numbers are the designations of envelope-associated PBPs of *C. crescentus* CB15 (left), envelope-associated and soluble PBPs of *C. crescentus* CB13 (middle) and *E. coli* PBPs (right). Cell envelopes of *E. coli* were prepared by the method described previously (Spratt, 1977a; Koyasu *et al.*, 1980a). (a) *C. crescentus* CB15 cell envelopes; (b) *C. crescentus* CB15 S100 fraction; (c) *C. crescentus* CB13 S100 fraction; (d, e) *C. crescentus* CB13 cell suspension; (f) *E. coli* cell envelopes.

Effect of temperature on soluble PBPs. One of the criteria for the characterization of PBPs is heat sensitivity (Nakagawa *et al.*, 1979; Noguchi *et al.*, 1979; Ohya *et al.*, 1979; Koyasu *et al.*, 1981). To examine differential temperature effects on the ability of PBPs to bind [^{14}C]penicillin G, the soluble S100 fraction was incubated at various temperatures for 10 min prior to PBP assay at $30\text{ }^{\circ}\text{C}$ (Fig. 4). PBP S2 was thermolabile and its penicillin-binding

Fig. 3. [¹⁴C]Penicillin G binding to PBPs. Soluble S100 fraction (a) or cell suspension (b) of *C. crescentus* CB13 were incubated with [¹⁴C]penicillin G at final concentrations of 0.01 to 300 μg ml⁻¹ at 30 °C for 10 min. The radioactivity bound to PBPs was estimated by densitometry of X-ray films and is plotted as a percentage of the radioactivity observed at 300 μg ml⁻¹. ○, PBP1A; ●, PBP1Bs; □, PBP2; ■, PBP3; △, PBP S1; ▲, PBP S2; ▽, PBP S3; ▼, PBP S4.

Fig. 4. Effect of temperature on soluble PBPs. The S100 fraction was pre-incubated at the indicated temperature for 10 min and then incubated with [¹⁴C]penicillin G at 30 °C. Slab gel electrophoresis and fluorography were carried out as described in Methods. The radioactivity of bound [¹⁴C]penicillin G was estimated by densitometry of the film and is plotted as a percentage of the radioactivity bound after pre-incubation at 30 °C for 10 min. ○, PBP S1; ●, PBP S2; □, PBP S3; ■, PBP S4.

activity was reduced to 40% after incubation at 40 °C for 10 min. The other PBPs were relatively thermostable, especially PBP S1 which was unaffected by incubation at 45 °C. PBP S3 and PBP S4 were less thermostable than PBP S1, and their activity was reduced to about 50% after incubation at 45 °C for 10 min – but residual activity of PBP S4 persisted even after incubation at 55 °C.

Fig. 5. Affinities of β -lactam antibiotics for soluble PBPs. Cell envelopes of *C. crescentus* CB15 (a) or the S100 fraction of *C. crescentus* CB13 (b–h) were incubated with antibiotics or distilled water by the pre-addition method. Antibiotic–PBP and [^{14} C]penicillin G–PBP complexes were separated by SDS–polyacrylamide gel electrophoresis (system 1). The numbers on the left are the designations of PBPs of the *C. crescentus* CB15 cell envelope. The numbers on the right are the designations of soluble PBPs of *C. crescentus* CB13. (a, b), no antibiotic addition; (c, d), apalcillin 0.24 and 6.0 $\mu\text{g ml}^{-1}$; (e, f), cephaloridine 0.19 and 4.7 $\mu\text{g ml}^{-1}$; (g, h), mecillinam 0.73 and 18 $\mu\text{g ml}^{-1}$.

Effect of related β -lactam antibiotics on soluble PBPs. Present information indicates that there is a close relationship between the affinity of β -lactam antibiotics for a particular PBP and their effect on cell morphology (Spratt, 1975; Curtis *et al.*, 1979; Noguchi *et al.*, 1979). As reported previously (Koyasu *et al.*, 1981), differential affinity of β -lactam antibiotics for PBPs in the *Caulobacter* cell envelope is quite distinct from that observed for PBPs in other bacteria. In particular, mecillinam, which induces ovoid cells in Gram-negative rod-shaped bacteria, did not show selective affinity for any of the *Caulobacter* envelope PBPs. In contrast, mecillinam has selective affinity for PBP2 of *E. coli* (Spratt & Pardee, 1975; Spratt, 1977a) and a single PBP in other bacteria (Curtis *et al.*, 1979; Noguchi *et al.*, 1979; Ohya *et al.*, 1979; Ogawara & Horikawa, 1980).

The effects on soluble PBPs of apalcillin, cephaloridine and mecillinam were examined by the pre-addition method (Koyasu *et al.*, 1981). The S100 fraction and a β -lactam antibiotic were mixed and incubated at 30 °C for 10 min prior to the addition of [^{14} C]penicillin G. Radioactivity bound to PBPs was detected by electrophoresis and subsequent fluorography. As shown in Fig. 5, the β -lactam antibiotics did not show a high affinity for the soluble PBPs, compared with the affinity for PBP1A and PBP1Bs in the cell envelope (Koyasu *et al.*, 1981). However, apalcillin exerted an effect on PBP S2 and PBP S3 at 6 $\mu\text{g ml}^{-1}$, while cephaloridine had little effect on the soluble PBPs. Mecillinam did not show selective binding to any of the soluble PBPs at a low concentration (0.73 $\mu\text{g ml}^{-1}$) although it exerted some effect on PBP S1 and PBP S2 at a high concentration (18 $\mu\text{g ml}^{-1}$). From this result and from previous observations (Koyasu *et al.*, 1981), it appears that *Caulobacter* does not possess a selective target for mecillinam either in the soluble fraction or in the cell envelope. PBP S4 was not affected by the β -lactam antibiotics tested, as was expected from the result that [^{14} C]penicillin G showed a very low affinity for this PBP (Fig. 3).

DISCUSSION

We have demonstrated the presence in the stalked bacterium *C. crescentus* of at least four PBPs in the soluble fraction, defined as the non-particulate fraction (S100) prepared as described in Methods. Our results suggest that the soluble PBPs are not the products of

proteolysis during preparation. In addition, the PBPs were observed in the intact cell (Fig. 2). It thus seems likely that the soluble PBPs are present in the cytoplasm and/or periplasm of the living cell. However, the possibility cannot be ruled out at the moment that the soluble PBPs are loosely bound to the envelope so as to be released during preparation, or that they are secreted specifically or cleaved by specific proteases *in vivo*. Whatever the precise explanation, additional PBPs may be present in the S100 fraction in other bacteria, and care should therefore be taken to analyse PBPs not only in the envelope but also in the soluble fraction.

PBP S1 and PBP S2 exhibited a penicillinase-like activity as assayed by the release of ^{14}C radioactivity from the [^{14}C]penicillin G–PBP complex. Since the patterns of the soluble PBPs changed little when the S100 fraction was incubated at 30 °C for 30 min prior to [^{14}C]penicillin G addition, the degradation of the PBPs during incubation appeared negligible. This penicillinase-like activity was similar to that of PBP5 and PBP6 of *E. coli* and low molecular weight PBPs of other bacteria (Shepherd *et al.*, 1977; Spratt, 1977*a*; Chase *et al.*, 1978; Noguchi *et al.*, 1979; Ohya *et al.*, 1979). Since *Caulobacter* does not possess low molecular weight PBPs which may correspond to *E. coli* PBP5 and PBP6 in its cell envelope (Koyasu *et al.*, 1980*a*, 1981), it is possible that PBP S1 and PBP S2 correspond to PBP5 and/or PBP6 of *E. coli*. It is known that *C. crescentus* possesses a penicillinase activity (Johnson & Ely, 1977) and is rather resistant to penicillin action. The relationship between the penicillinase activity and the soluble PBPs remains to be investigated.

Caulobacter crescentus CB13 does not possess PBP4 in its envelope, and in *C. crescentus* CB15 this PBP is located in the outer membrane (Koyasu *et al.*, 1980*a*, 1981). By contrast, as shown in Fig. 2, *C. crescentus* CB15 did not possess PBP S2. The heat sensitivity of PBP S2 of *C. crescentus* CB13 was similar to that of PBP4 of *C. crescentus* CB15 (Fig. 4; Koyasu *et al.*, 1981). However, PBP4 of *C. crescentus* CB15 did not show penicillinase-like activity (Koyasu *et al.*, 1981) nor did it co-migrate with PBP S2 of *C. crescentus* CB13 (Fig. 2; Fig. 5). [^{14}C]Penicillin G showed much higher affinity for PBP S2 than for PBP4. These findings suggest that PBP S2 is a different PBP from PBP4. Further studies should serve to clarify the relation of these PBPs.

Both soluble (Fig. 4) and envelope-associated (Koyasu *et al.*, 1981) PBPs of *Caulobacter* were heat labile compared with those of *E. coli* (Nakagawa *et al.*, 1979). PBP3 and PBP S2 were so heat labile that their ability to bind [^{14}C]penicillin G was lost during incubation at 40 °C for 10 min.

One approach to elucidate the functions of PBPs is to investigate the relationship between effects on cell morphology and the affinity for PBPs of different β -lactam antibiotics. In a previous study (Koyasu *et al.*, 1981), it was suggested, from the effect of some β -lactam antibiotics on PBPs and cell morphology, that PBP3 of the *C. crescentus* cell envelope is involved in cell division. Similar use of β -lactam antibiotics did not indicate any specificity of the drugs on soluble PBPs. Mecillinam did not show a specific effect on any of the PBPs in either soluble (Fig. 5) or envelope (Koyasu *et al.*, 1981) fractions. Since *C. crescentus* is resistant to a high concentration of mecillinam, it seems that this organism does not have a specific target for this antibiotic.

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