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Penicillin-Binding Site on the *Escherichia coli* Cell Envelope

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The binding of ^{35}S-labeled penicillin to distinct penicillin-binding proteins (PBPs) of the "cell envelope" obtained from the sonication of *Escherichia coli* was studied at different pHs ranging from 4 to 11. At low pH, PBPs 1b, 1c, 2, and 3 demonstrated the greatest amount of binding. At high pH, these PBPs bound the least amount of penicillin. PBPs 1a and 5/6 exhibited the greatest amount of binding at pH 10 and the least amount at pH 4. With the exception of PBP 5/6, the effect of pH on the binding of penicillin was direct. Experiments distinguishing the effect of pH on penicillin binding by PBP 5/6 from its effect on beta-lactamase activity indicated that although substantial binding occurred at the lowest pH (4), the amount of binding increased with pH, reaching a maximum at pH 10. Based on earlier studies, it is proposed that the binding at high pH involves the formation of a covalent bond between the C-7 of penicillin and free epsilon amino groups of the PBPs. At pHs ranging from 4 to 8, position 1 of penicillin, occupied by sulfur, is considered to be the site that establishes a covalent bond with the sulfhydryl groups of PBP 5. The use of specific blockers of free epsilon amino groups or sulfhydryl groups indicated that wherever the presence of each had little or no effect on the binding of penicillin by PBP 5, the presence of both completely prevented binding. The specific blocker of the hydroxyl group of serine did not affect the binding of penicillin. These observations suggest that a molecule of penicillin forms simultaneous bonds between its S at position 1 and sulfhydryl groups of PBP 5 and between its C-7 and free epsilon amino groups of PBP 5.

The determination of penicillin-binding proteins (PBPs) present on the cytoplasmic membrane of bacteria is accomplished by the incubation of "cell envelopes" obtained from disrupted bacteria with radioactively labeled benzylpenicillin, followed by acrylamide gel electrophoresis and autoradiography (5). The conditions used for this assay are more or less constant from laboratory to laboratory with respect to pH, which is generally maintained at 7. The comprehensive studies reviewed by Parker et al. (4) show that the binding of penicillin to protein, the derivative of penicillin bound to protein, and the molecular group of the protein to which binding occurs are markedly affected by pH. Conditions which favor the depression of ionization of the epsilon amino groups of the amino acids of the protein favor the covalent binding of penicillin to the epsilon amino groups. Consequently, as the pH of the milieu is increased, more of the penicillin is bound to the epsilon amino groups of basic amino acids of the protein. In addition, the component of penicillin which is involved in a covalent bond with epsilon amino groups is C-7. At pHs ranging from 4 through 8, penicillin forms a disulfide bond with the sulfhydryl group of the protein.

The studies cited above suggest that similar reactions between penicillin and the PBPs may readily occur in accordance with pH. No comprehensive studies have been reported which investigated the effect of pH on the binding of penicillin to distinct PBPs of the gram-negative bacterium. The present study was concerned with this effect.

MATERIALS AND METHODS

E. coli PA3092 (characterized and maintained by U. Schwarz at the Max Planck Institute), requiring lysine, diaminopimelic acid, and thymidine, was cultured overnight at 37°C in Penassay broth (Difco Laboratories) supplemented with lysine, diaminopimelic acid, and thymidine at concentrations of 20, 20, and 50 μg/ml of medium, respectively. Samples (10 ml) of these cultures were transferred to 1-liter flasks containing Penassay broth supplemented as above, and the cultures were incubated at 37°C in a shaking incubator. When the culture reached an optical density of 0.6 at 578 nm, it was centrifuged at 6,000 × *g* for 10 min and suspended in 0.01 M Tris maleate (pH 8.0) (5). The concentration of the sedimented cultures was adjusted to 0.4 g/ml of medium, and the suspensions were supplemented with 40 μg of DNase (Sigma Chemical Co.) per ml. The suspensions were sonicated at 100 W for three 60-s bursts, with each burst followed by 90 s of rest. Sonication was performed in an ice bath maintained at 4°C. The products of sonication were examined by phase microscopy for the determination of cell breakage. Under the conditions described, over 90% of the cells were disrupted. The sonicated products of the disrupted bacteria are termed cell envelopes throughout the text.

The products of cell disruption, suspended in Tris maleate buffer, were centrifuged at 60,000 × *g* for 30 min, and the pellets were suspended in 0.01 M K_2PO_4 (pH 7.0). The protein concentration of each preparation of cell envelopes was adjusted to 10 mg/ml after determination of total protein by the method of Lowry et al. (3). The determination of PBPs of the cell envelope was accomplished by the method of Spratt (5). Briefly, 20 μg of cell wall envelopes (20 μl) was incubated with 30 ng of ^{35}S-labeled benzylpenicillin (volume, 5 μl; specific activity, 1.6 Ci/mmol; New England Nuclear Corp.) for 10 min at 30°C; 10 μl of 20% Sarkosyl (Sigma) was then added and mixed, 150 μg (5 μl) of nonradioactive benzylpenicillin (Sigma) was added, and the tubes were incubated for an additional 20 min at room temperature. The incubation products were centrifuged at 100,000 × *g* for 10 min, and the supernatants were transferred to tubes contain-

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TABLE 1. Effect of pH on binding of [^{35}S]penicillin by PBPs of *E. coli*

pH	Amt (U) of [^{35}S]penicillin bound[a] by PBP:						
	1a	1b	1c	2	3	4	5/6
4	280	444	568	450	228	444	3,401
5	375	280	604	506	409	512	3,658
6	384	220	330	389	267	608	3,758
7	402	213	249	143	285	446	4,205
8	441	156	65	70	121	353	4,492
9	680	103	90	90	132	370	5,538
10	1,049	107	107	26	115	317	5,866
11	828	118	120	0	158	336	5,456

[a] Amount of binding in arbitrary units derived from the relative optical extinction of each autoradiographic band of the film, as determined with the aid of a computer-assisted Beckman CDS-200 integrating densitometer.

ing 20 μl of Spratt buffer (50 mM Tris hydrochloride [pH 7.2], 50% [wt/vol] glycerol, 5% [wt/vol] sodium dodecyl sulfate, 0.01% [wt/vol] bromophenol blue) and 5 μl of mercaptoethanol. The tubes were placed in a boiling water bath for 3 min, and then 50 μl of the contents was electrophoresed on 10% acrylamide (10% sodium dodecyl sulfate) slab gels. The gels were stained with Coomassie blue, destained overnight in 5% methanol–7.5% acetic acid, placed onto prewetted Whatman no. 1 MM filter paper, dried under vacuum and heat, and incubated with X-ray film (Eastman Kodak Co.) for 9 days at −70°C. The exposed films were developed, and the autoradiographic bands were quantified with the aid of a computer-assisted Beckman CDS-200 integrating densitometer. The data presented are in arbitrary units of relative extinction automatically calculated by the instrument. For those experiments involving different pHs for either the binding incubation or postbinding incubations, details are presented below in the appropriate sections.

All experiments described were repeated three times. The qualitative results were essentially similar for all cases examined.

Purified PBP 5 (95% pure; 5% impurity due to presence of PBP 6) from *E. coli* PA3092 was generously provided by W. Keck of the Max Planck Institute. The binding of ^{35}S-labeled penicillin to PBP 5 was determined in the absence and presence of a variety of free epsilon amino group blockers and sulfhydryl group blockers, either singly or in combination. The details of the binding conditions are described in the legend to Fig. 1.

RESULTS

The binding of [^{35}S]penicillin by the PBPs of the *E. coli* cell envelope at pH 7 in terms of the relative optical extinction properties of the autoradiographic bands of the film is presented in Table 1. The major binder of penicillin was PBP 5/6 (under the conditions used, PBPs 5 and 6 were not readily separated from each other) and accounted for approximately 70% of the total amount of [^{35}S]penicillin bound. The effect of pH on the amount of penicillin bound by PBP 5 was substantial. The least amount of penicillin bound by PBP 5/6 occurred at pH 4, whereas the maximum amount bound occurred at pH 10. At this latter pH, the amount bound was approximately 40% greater than that bound at pH 7. The amount of [^{35}S]penicillin bound by PBP 1a was similarly affected by pH. At a pH of 4 the least amount of binding occurred, whereas maximum binding was evident at a pH of 10. The amount of penicillin bound at pH 10 was approximately 2.6 times greater than that bound at pH 7. Unlike PBPs 5/6 and 1a, PBPs 1b, 1c, and 2 demonstrated greater binding activity at low pH. Increasing the pH resulted in appreciable decreases in the binding of penicillin. Of particular interest is the effect of pH on the binding of penicillin by PBP 2. Maximum binding occurred at pH 5. At pH 7, the amount of binding was reduced by close to 70%. Little or no binding of penicillin to PBP 2 was detected at pH 11. The effect of pH on the binding of penicillin by PBP 3 was similar to that observed for PBPs 1b, 1c, and 2. PBP 4 appeared to be least affected by pH.

The binding of [^{35}S]penicillin to almost all of the PBPs of the *E. coli* cell envelope was markedly affected by pH. Whether pH has an effect on the retention of penicillin bound to each of the PBPs was of obvious interest, especially with respect to PBP 5, a molecule with demonstrated beta-lactamase activity (1). To determine this, the binding of penicillin was performed at pH 7 for 10 min and the cell envelopes were centrifuged at 10,000 × *g*, washed twice with buffer (pH 7), and suspended in buffers with pHs ranging from 4 to 11. The cell envelopes were incubated for an additional 20 min, and nonradioactive penicillin and Sarkosyl were then added. A duplicate set was processed after the initial incubation and served as the controls for detecting the amount of penicillin bound per PBP before the suspension of the cell envelopes at different pHs. The results (Table 2) indicate that the amount of penicillin bound by each of the PBPs, with the exception of PBP 5/6, remained unchanged. In other words, once penicillin was bound to PBPs other than PBP 5/6, the alteration of pH had no detectable effect on the amount of penicillin retained. The amount of penicillin retained by PBP 5 was affected by pH. At the altered pHs of 7 and 8, the amount of penicillin retained was the least. At the higher pH the amount of penicillin associated with PBP 5/6 was greatest.

The effect of pH 7 on the retention of penicillin bound at different pHs was similarly studied. For these experiments the binding of penicillin was performed at different pHs in duplicate. One set was treated as described in Materials and Methods; the second set was washed twice with buffer, pH 7, and the cell envelopes were resuspended in the same buffer (pH 7) and incubated for an additional 20 min. The data (Table 3) indicate that, with the exception of PBP 5/6, the retention of penicillin initially bound to each PBP at different pHs was unaffected by the additional 20 min of incubation at pH 7. Unlike the other PBPs, the amount of penicillin retained by PBP 5/6 was markedly affected by the 20-min incubation period; the amount of penicillin bound at each pH decreased by over 70% by the end of the additional 20-min incubation at pH 7.

TABLE 2. Effect of pH on retention of penicillin bound by PBPs of *E. coli* cell envelope at pH 7

pH	Amt (U) of [^{35}S]penicillin bound by PBP[a]:						
	1a	1b	1c	2	3	4	5/6
4	456	199	255	161	313	516	4,785
5	401	218	240	129	288	483	4,870
6	847	176	283	119	307	456	3,933
7	451	194	267	155	299	480	3,401
8	444	208	276	134	251	509	3,560
9	475	188	238	148	280	472	4,835
10	470	226	229	151	301	501	4,726
11	426	201	254	156	279	475	4,699

[a] Amount (units) of penicillin bound at pH 7 before incubation at different pHs was as follows: 1a, 476; 1b, 224; 1c, 289; 2, 129; 3, 345; 4, 474; 5/6, 4,754.

TABLE 3. Effect of pH 7 on retention of penicillin bound by PBPs of cell envelope at different pHs

Binding pH	Amt (U) of [^{35}S]penicillin bound before/after additional incubation at pH 7 by PBP:						
	1a	1b	1c	2	3	4	5/6
4	289/255	416/391	470/494	401/419	180/196	380/389	3,076/911
5	265/290	333/300	549/530	459/436	357/370	419/399	3,351/936
6	360/346	164/175	316/301	386/411	301/288	509/555	3,924/987
7	298/359	187/170	320/271	219/200	266/283	500/475	4,442/1,191
8	386/430	140/118	115/100	110/93	140/116	403/414	4,816/1,301
9	519/498	106/96	51/70	79/95	109/119	403/400	5,219/1,481
10	926/898	31/240	51/68	8/11	115/108	350/338	5,553/1,515
11	751/770	30/37	46/68	8/12	96/105	322/279	4,817/1,451

The data (Table 3) also indicate that after 20 min of additional incubation at pH 7, the residual amount of penicillin bound was least at pH 4 and greatest at the higher pHs. These data, therefore, suggest that for PBP 5, the binding of penicillin is pH determined. Furthermore, although the least amount of binding occurred at pH 4, binding was still substantial when compared to the other pHs. This suggests, therefore, that the binding of penicillin to PBP 5, evident at the pH extremes, is probably bimodal, that is, different free reactive groups of the amino acids of PBP 5 are involved in the binding of penicillin and the extreme pHs ultimately control which amino acids bind penicillin. To determine the validity of this concept, purified PBP 5 obtained from *E. coli* PA3092 was assayed for penicillin-binding activity in the absence or presence of various specific blockers of amino and sulfhydryl groups used singly or in combination (Fig. 1). The presence of phthalmic anhydride, a blocker of -NH_2 groups, or *p*-chloromercuribenzoate, a blocker of sulfhydryl groups, marginally reduced the amount of penicillin bound. The presence of the -NH_2 blocker succinic anhydride, the sulfhydryl blocker iodoacetate, or the -OH blocker of serine, *p*-nitrophenylacetate, at the concentrations used had no obvious detectable effect on the binding of penicillin. However, the simultaneous presence of either -NH_2 blocker and either -SH blocker prevented the binding of penicillin to PBP 5. Neither the electrophoretic migration of PBP 5 nor its staining characteristics revealed any changes promoted by the presence of the various blockers themselves. This suggests that at the concentrations used, the PBP 5 molecule remained intact.

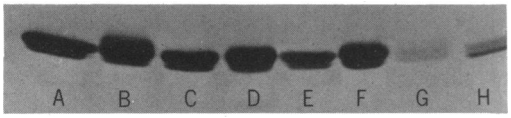

FIG. 1. Binding of [^{35}S]penicillin by purified PBP 5 of *E. coli* PA3092. Purified PBP 5 (100 μg) from *E. coli* PA3092 was incubated with [^{35}S]penicillin, as described in Materials and Methods, for 15 min in the absence and presence of blockers of free epsilon amino groups (succinic anhydride or phthalmic anhydride), blockers of sulfhhydryl groups (iodoacetate or *p*-chloromercuribenzoate), the blocker of the hydroxyl group of serine, *p*-nitrophenylacetate, or combinations of amino and sulfhydryl group blockers. The remainder of the penicillin-binding assay was conducted as described in Materials and Methods. Because of the great intensity of the autoradiographic bands, the use of densitometric analysis was not readily possible. Neither the electrophoretic properties nor the staining characteristics of PBP 5 were altered by the presence of the blockers at the concentrations indicated. Lanes: A, control; B, succinic anhydride (40 μg/ml); C, phthalmic anhydride (40 μg/ml); D, iodoacetate (40 μg/ml); E, *p*-chloromercuribenzoate (40 μg/ml); F, *p*-nitrophenylacetate (40 μg/ml); G, *p*-chloromercuribenzoate (20 μg/ml) plus succinic anhydride (20 μg/ml); H, *p*-chloromercuribenzoate (20 μg/ml) plus phthalmic anhydride (20 μg/ml).

DISCUSSION

It has been shown (4) that the binding of penicillin to protein at high pH results in the formation of a covalent bond between C-7 of penicillin and the free epsilon NH_2 group of the protein moiety (lysine). At low pH, penicillin is converted rapidly to penicillenic acid, which then establishes a covalent bond between its S at position 1 and free SH groups of the protein (cystine) (3). The resulting penicillin is termed penicillenate. Although penicillin can be converted to penicillenic acid at pHs above 7, penicillenate does not readily form at pHs above 8. Therefore, above a pH of 8, disulfide bonding between the S at position 1 of penicillin and the -SH group of the protein does not occur.

The above studies, conducted and reviewed by Parker et al. (4), indicate that the pH of the system controls the type of covalent bond that occurs between penicillin and potentially reactive groups of the protein. The results presented in this study indicate that the amount of penicillin bound to each PBP of the *E. coli* cell envelope is markedly affected by pH.

For PBP 5 the amount of penicillin bound is the net result of the effect of pH on the binding and beta-lactamase activity of this PBP during the incubation period. Some understanding of the contribution of beta-lactamase activity subsequent to the initial binding of penicillin at various pHs is afforded by the data in Table 3. The 20 min of additional incubation at pH 7 allowed beta-lactamase activity to proceed. The amount of penicillin retained at the end of this period provides some indication of the extent of binding at various pHs. Assuming that beta-lactamase activity was not altered by the initial pHs used during the binding of penicillin, the amount of penicillin bound increased with pH. Since binding was substantial at the lowest pH used, namely pH 4, and greatest at pH 10, the binding of penicillin to PBP 5 must have involved at least two reactive sites of the molecule. At low pH, the formation of penicillenic acid is anticipated and the ensuing disulfide bond between the S of penicillin and an -SH group of PBP 5 is favored. At pHs above 8, disulfide bonding could not occur. However, above this pH, the formation of a covalent bond between C-7 of penicillin and epsilon amino groups of PBP 5 (lysine) would be favored. At pHs 7 to 8 both types of bonds would be expected provided that the inductive effects of neighboring amino acids depressed the pK_2(E-NH_2) of lysine.

The formation of covalent bonds between penicillin and epsilon amino groups and -SH groups of PBP 5 is inferred because the simultaneous presence of the blockers of these groups at neutral pH obviated the binding of penicillin. Since the presence of these blockers did not alter the electropho-

retic properties of PBP 5, the elimination of penicillin binding may be safely considered to be due to direct blocking of the groups. Furthermore, because the individual blockers of SH or epsilon amino groups did not substantially prevent binding but binding was completely blocked by their simultaneous presence, it is further suggested that a molecule of penicillin simultaneously establishes two types of bonds with PBP 5. It is envisioned that this simultaneous bond formation results in a link between C-7 of penicillin and the epsilon amino groups of the lysine of PBP 5 and a link between the S at position 1 and the sulfhydryl group of cystine. This may account for the previous failure of others to reduce the binding of penicillin by the use of sulfhydryl blockers alone (1). In addition, since the blocker of serine did not affect the binding of penicillin to PBP 5 and since the simultaneous presence of epsilon amino group and sulfhydryl group blockers completely prevented penicillin binding by PBP 5, the binding of penicillin by the OH group of serine, as shown by others (2) for D-alanyl-carboxypeptidase of *Streptomyces* strain R61, may be ruled out.

With the exception of PBP 2, all of the PBPs bound penicillin from pHs 4 through 11. This suggests that these PBPs bind penicillin in a manner similar to that postulated for PBP 5. For PBP 2, the binding site of penicillin may be completely restricted to the formation of disulfide bonds.

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