Biochem. J. (1987) 247, 29–33 (Printed in Great Britain)

Inactivation of the thiol RTEM-1 β-lactamase by 6-β-bromopenicillanic acid

Identity of the primary active-site nucleophile

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The thiol RTEM-1 β-lactamase [Sigal, Harwood & Arentzen (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7157–7160] is inactivated by 6-β-bromopenicillanic acid with formation of a characteristic chromophore, absorbing maximally at 350 nm, which is covalently bound to the enzyme. Model studies suggest that the chromophore is that of a 6-carboxylate thiol ester of 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylate, which can arise by rearrangement of the thiol-penicilloate obtained by thiolysis of the β-lactam of 6-β-bromopenicillanate. Loss of activity of the enzyme is also concerted with disappearance of its single (cysteine) thiol group. These results indicate that the thiol group of this enzyme is indeed a nucleophilic catalyst in β-lactam turnover. The thiol β-lactamase is also inactivated by clavulanic acid with formation of a chromophore, presumably a 3-aminoacrylate thiol ester, at 308 nm. Both 6-β-bromopenicillanate and clavulanate are hydrolysed more slowly by the thiol enzyme than by the native serine β-lactamase, but, probably as a consequence of this, both compounds inactivate the former enzyme more efficiently. Cefoxitin, a poor substrate of the native enzyme, does not appear to interact covalently with the thiol β-lactamase.

INTRODUCTION

Class A and class C β-lactamases catalyse the hydrolysis of β-lactams by a double-displacement mechanism, where the primary nucleophile is a serine hydroxy group [1–4]. Although there has been speculation concerning the existence of thiol β-lactamases [5], where a cysteine thiol group would replace the serine hydroxy group as the primary active-site nucleophile, no naturally occurring examples have yet been characterized. The existence of thiol proteinases as well as serine proteinases, however, suggests that an effective thiol β-lactamase could have evolved, or, if it did not, could be designed.

The closest approximation to a thiol β-lactamase currently available is the enzyme prepared by Sigal *et al.* [6] from the RTEM-1 β-lactamase, where the active-site serine residue (serine-70 [5]) has been replaced by a cysteine through site-specific mutagenesis. It is only an approximation, of course, because the rest of the protein, and in particular the other residues making up the active site, remain, in principle at least, optimal (and close to perfect [7–9]) for a serine β-lactamase. That this is a significant distinction is indicated by the much poorer performance of the mutant enzyme as a β-lactamase [10]. A similar loss in activity towards specific substrates has been observed on this transformation of serine proteinases [11–13]. The precise reasons, at the molecular level, for this loss are not agreed upon [11,12,14–17].

We have begun mechanistic studies with the thiol RTEM-1 β-lactamase described by Sigal *et al.* [6] in order to gain perspective into the auxiliary functionality of the β-lactamase active site, and perhaps also into the source of the diminished activity mentioned above. In this initial paper we report evidence that the cysteine-70 thiol group of the thiol RTEM-1 β-lactamase is indeed a nucleophilic catalyst in β-lactam turnover.

EXPERIMENTAL

Materials

The HB101 strain of *Escherichia coli* K12 containing the pOTBL plasmid [10] was generously provided by E.I. du Pont de Nemours and Co. The RTEM-2 β-lactamase was purchased from the P.H.L.S. Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K., and used as received. Sodium 6-β-bromopenicillanate, sodium clavulanate, cephaloridine and nitrocefin were generously given by Leo Pharmaceutical Products, Beecham Pharmaceuticals, Eli Lilly and Co. and Glaxo Research respectively. Benzylpenicillin was purchased from Sigma Chemical Co.

Preparation and purification of the thiol RTEM-1 β-lactamase

Cells (pOTBL/HB101) were grown to late exponential phase at 37 °C in LB medium [200 litres with 0.9 MPa (120 lbf/in²) aeration] containing ampicillin (5 mg/l), harvested, and resuspended in 200 litres of M9CA medium [18] at 37 °C with 1.1 MPa (160 lbf/in²) aeration. In the latter medium, induction and expression of the thiol β-lactamase were allowed to proceed for 2.5 h before the cells were harvested by centrifugation at 4 °C. All subsequent manipulation was done at 4 °C.

The harvested cell paste (1300 g) was washed with a buffer of pH 8.0 containing 50 mM-Tris, 20 mM-EDTA and 20% (w/v) sucrose. Cells were centrifuged at 5000 *g* for 10 min, and the wash was repeated twice

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more. The combined wash solution (approx. 1 litre) was dialysed against a buffer of pH 7.8 containing 50 mM-Tris, 20 mM-EDTA, 6 mM-2-mercaptoethanol and 0.01% NaN_3, and then concentrated to 300 ml with an Amicon YM-10 filter. The concentrate was dialysed against 25 mM-imidazole buffer at pH 7.4, also containing 3 mM-2-mercaptoethanol, concentrated to 250 ml, and divided into 25 ml portions for storage under N_2 at −70 °C. In this form the enzyme appeared to be stable for months.

Further purification involved chromatofocusing and then Sephadex G-75 chromatography, as described by Sigal *et al.* [10]. Each 25 ml portion yielded 8–10 mg of the thiol β-lactamase, which was pure by SDS/polyacrylamide-gel electrophoresis and had a specific activity of about 7.5 units (μmol/min)/mg against 0.14 mM-nitrocefin. In this form [in 50 mM-Tris/0.1 M-NaCl/3 mM-2-mercaptoethanol/0.01% NaN_3/1 mM-EDTA, pH 7.2 (T buffer)] the β-lactamase activity was generally stable for 2–3 weeks when kept under N_2 at 4 °C.

Synthesis of sodium 2,3-dihydro-2,2-dimethyl-6-thiomethoxycarbonyl-1,4-thiazine-3-carboxylate (I)

Sodium metal (110 mg, 4.8 mmol) was added in portions to molten (43 °C) phenol (500 mg, 5.3 mmol) under a stream of N_2, yielding, after reaction, a precipitate of sodium phenoxide. This mixture was dissolved in 1 ml of anhydrous dimethylformamide (distilled from BaO) and added dropwise to a stirred solution of the sodium salt of 6-β-bromopenicillanic acid (100 mg, 0.34 mmol) in 5 ml of anhydrous dimethylformamide, also kept under an atmosphere of N_2. After 6 h at room temperature, the solvent was removed under reduced pressure. The residue was dissolved in water, and the solution was extracted with dichloromethane until the excess phenol was removed. The aqueous layer was then freeze-dried, yielding the sodium salt of 2,3-dihydro-2,2-dimethyl-6-phenoxycarbonyl-1,4-thiazine-3-carboxylic acid (88 mg, 0.28 mmol, 83%), which was characterized by its ^{1}H-n.m.r. spectrum [^2H_2O, δ (p.p.m.) 1.33 (3H, s, 2-CH_3), 1.53 (3H, s, 2-CH_3), 3.93 (1H, s, 3-*H*), 7.15–7.50 (5H, m, Ar*H*) and 8.03 (1H, s, 5-*H*)] and absorption [water, $\lambda_{max.}$ 318 nm (ϵ = 10200 $M^{-1}\cdot cm^{-1}$)] spectrum.

The phenyl ester (10 mg, 0.032 mmol) and a 10-fold excess of sodium thiomethoxide (Aldrich Chemical Co.) were suspended in 2 ml of anhydrous dimethylformamide and heated under reflux for 15 min under an atmosphere of N_2. The solvent was removed under reduced pressure, and the residue was taken up into water. After the solution had been extracted several times with dichloromethane, it was freeze-dried, yielding the crude sodium salt of the product. This material was taken up into the minimum volume of water, applied to a Sephadex G-10–120 (1 cm × 18 cm) column, and eluted with water. Fractions containing the product were identified by absorption spectra, pooled, and freeze-dried, yielding 5.6 mg (0.021 mmol, 66%) of the required product, which was characterized by its ^{1}H-n.m.r. spectrum [^2H_2O, δ (p.p.m.) 1.27 (3H, s, 2-CH_3), 1.49 (3H, s, 2-CH_3), 2.34 (3H, s, –SCH_3), 3.90 (1H, s, 3-*H*) and 8.01 (1H, s, 5-*H*)], i.r. spectrum [KBr, 3460(NH), 1621 and 1558 cm^{-1}) and absorption spectrum [T buffer, $\lambda_{max.}$ 345 nm (ϵ = 9100 $M^{-1}\cdot cm^{-1}$)] spectra.

Analytical methods

Thiol RTEM-1 β-lactamase and native RTEM-2 β-lactamase concentrations were determined spectrophotometrically, by using the absorption coefficient of the RTEM-2 enzyme, 29400 $M^{-1}\cdot cm^{-1}$ at 281 nm [19]. Routine assays of the thiol RTEM-1 β-lactamase activity were carried out spectrophotometrically at 232 nm against 0.5 mM-benzylpenicillin in a buffer of pH 7.5 containing 0.1 M-phosphate, 1 mM-EDTA and 3 mM-2-mercaptoethanol at 25 °C. The wild-type enzyme (RTEM-2) was assayed spectrophotometrically at 260 nm against 0.1 mM-cephaloridine in 0.1 M-phosphate buffer at pH 7.5 and at a temperature of 25 °C.

Thiol titration

The free thiol content of enzyme samples was determined spectrophotometrically by titration with 4,4′-dipyridyl disulphide. Solutions of 4,4′-dipyridyl disulphide (approx. 0.6 mM) in buffer were prepared as described by Brocklehurst & Little [20]. Enzyme samples were freed of 2-mercaptoethanol by dialysis against 50 mM-Tris/0.1 M-NaCl/1 mM-EDTA buffer at pH 7.2. A sample of the enzyme solution (generally 50 μl, leading to a final enzyme concentration of between 1 and 2 μM) was then added to 0.5 ml of the disulphide solution, and the increase in absorption at 324 nm was measured. Thiol concentrations could then be calculated by using 19800 $M^{-1}\cdot cm^{-1}$ as the absorption coefficient of pyrid-4-thione at that wavelength [20].

Reaction of the thiol β-lactamase with 6-β-bromopenicillanate

Small portions of a 6-β-bromopenicillanate solution were added to samples (100 μl) of a thiol RTEM-1 β-lactamase solution (28 μM) in T buffer, such that the final concentrations of 6-β-bromopenicillanate varied from zero to 30 μM. After 7 h at 4 °C these mixtures were assayed for β-lactamase activity against benzylpenicillin and, after removal of the 2-mercaptoethanol by dialysis, absorption spectra were recorded and the thiol content was determined by using 4,4′-dipyridyl disulphide as described above.

Isolation of the inactivated thiol β-lactamase

6-β-Bromopenicillanate was added (final concentration 32 μM) to 0.5 ml of a 31 μM solution of the thiol β-lactamase in T buffer in a cuvette, and the absorption spectrum was monitored with time. After formation of the 350 nm chromophore was complete, a β-lactamase assay against benzylpenicillin showed that all β-lactamase activity had been lost; a further addition of 6-β-bromopenicillanate led to no additional absorption at 350 nm. The inhibited enzyme was then isolated by Bio-Rad P2DG column (1 cm × 10 cm) chromatography on elution with water or T buffer. Absorption spectra were recorded directly from the column and after addition of guanidinium chloride to 6 M.

Kinetics of reaction of the thiol β-lactamase with 6-β-bromopenicillanate

Pseudo-first-order rates of reaction of the thiol enzyme with 6-β-bromopenicillanate were determined spectrophotometrically at 350 nm. Reaction mixtures consisted of enzyme (10 μM) and 6-β-bromopenicillanate (0.2–

1.0 mM) in T buffer at 25 °C. Pseudo-first-order rate constants were calculated from the plots of absorption versus time.

Reaction of the thiol β-lactamase with clavulanate

This was studied essentially as described above for 6-β-bromopenicillanate. Absorption spectra and β-lactamase activities were measured on addition of up to 8 equivalents of clavulanate.

Reaction of the wild-type RTEM-2 β-lactamase with 6-β-bromopenicillanate

These experiments were also carried out as those described above. Rates of reaction were measured spectrophotometrically at 306 nm.

RESULTS AND DISCUSSION

The basic procedure of Sigal *et al.* [10] for growth of the cells and purification of the enzyme was followed. Slight changes to the procedures involved, firstly, growth of the cells in batch rather than continuous culture. Secondly, 20% sucrose was included in the EDTA wash that released the periplasmic enzyme, in order to keep the cells as intact as possible. Preliminary experiments showed that this procedure released purer enzyme. The remaining enzyme, more strongly bound to the cells, making up, by our procedure, only about 10% of the total activity, and which Sigal *et al.* [10] released by lysozyme treatment, we chose to leave because of the small quantity involved, its much lesser purity and subsequently longer purification procedure, and because preliminary experiments showed that this material often lost activity during the purification. Finally, it was found that, during the purification, the enzyme could be stored more stably in imidazole buffer before chromatography.

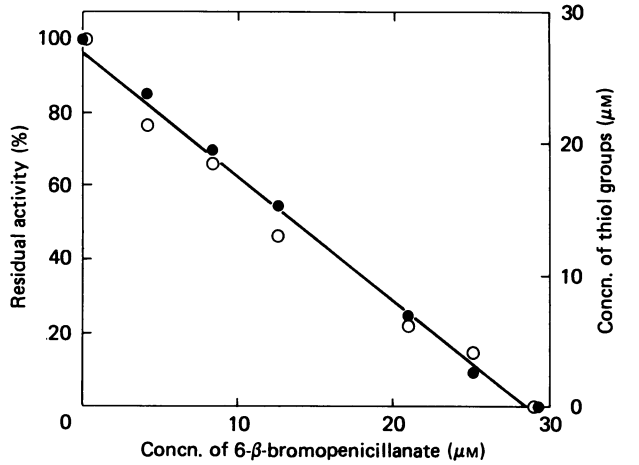


Fig. 1. Residual β-lactamase activity against benzylpenicillin (●) after incubation of the thiol RTEM-1 β-lactamase (28.0 μM) with various concentrations of 6-β-bromopenicillanate

Also shown is the residual thiol content of the enzyme (○), by 4,4′-dipyridyl disulphide titration, as a function of 6-β-bromopenicillanate concentration. A least-squares line is drawn through all of the points.

All of our enzyme was stored this way and it was purified in smaller batches (8–10 mg) as needed. Despite these changes, the yield and specific activity of the pure enzyme were essentially identical with those reported by Sigal *et al.* [10]. As also reported by Sigal *et al.* [10], the purified enzyme contained one free thiol group per molecule, determined by titration with 4,4′-dipyridyl disulphide, and was completely inactivated by 1.5 equivalents of *p*-hydroxymercuribenzoate.

The thiol β-lactamase was also very sensitive to the class A (including RTEM-2) β-lactamase inhibitor 6-β-bromopenicillanic acid [21]. Fig. 1 shows the results of a titration of this reagent against the thiol β-lactamase. It is clear from the intercept on the abscissa that 6-β-bromopenicillanate inactivated the enzyme on 1:1 interaction; that essentially no turnover occurred was also evident from the absence of an absorption peak at 306 nm in spectra of these reaction mixtures, which would correspond to generation of the hydrolysis and rearrangement product (IV) (see below). Also plotted in Fig. 1 is the thiol content of the enzyme, derived from 4,4′-dipyridyl disulphide titration, as a function of the amount of 6-β-bromopenicillanate added. The loss of activity on reaction with 6-β-bromopenicillanate directly correlates with the loss of the free thiol group. This suggests, as would be expected for an enzyme thought to be a thiol β-lactamase, that the thiol group is essential for activity.

The nature of the interaction between the thiol group and the inhibitor was investigated by absorption spectroscopy. Fig. 2 shows the absorption spectrum of the isolated (by gel filtration) inactivated enzyme in the absence and in the presence of 6 M-guanidinium chloride. The presence of the unusual chromophore at 350 nm (which moved to 345 nm in guanidinium chloride) is a clear indication that the inhibitor remained covalently bound to the enzyme. Under the same conditions the model compound (I) exhibited an absorption maximum at 345 nm with absorption coefficient 9100 $M^{-1} \cdot cm^{-1}$. This spectrum was essentially unchanged in 6 M-guanidinium chloride.

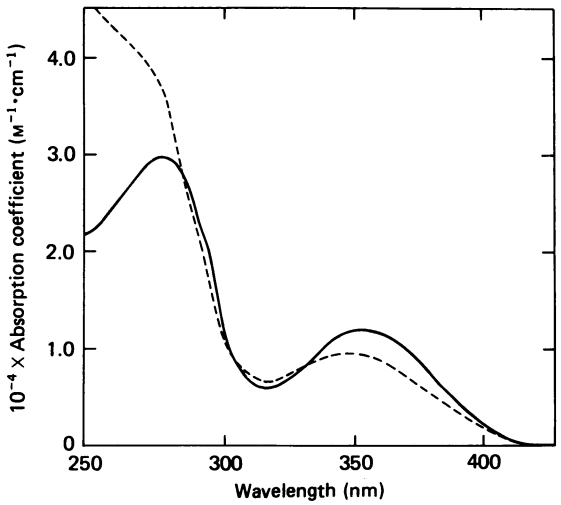
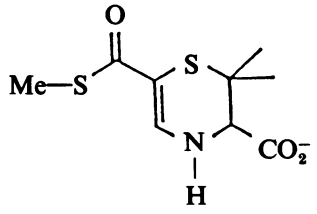
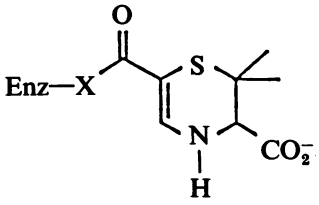


Fig. 2. Absorption spectra of the 6-β-bromopenicillanate-inactivated thiol RTEM-1 β-lactamase in the absence (——) and in the presence (– – –) of 6 M-guanidinium chloride



(I)

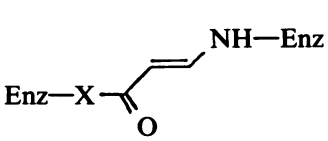
These results suggest that the structure of the covalently bound inhibitor in the inactive complex is (II) (X = S). This conclusion is of course also strongly supported by previous work with 6-β-bromopenicillanic acid and *Bacillus cereus* β-lactamase I, a class A β-lactamase, where the structure of the bound inhibitor was shown to be (II) (X = O) [2,22]. The latter result has recently been generalized to a broad range of serine β-



(II)

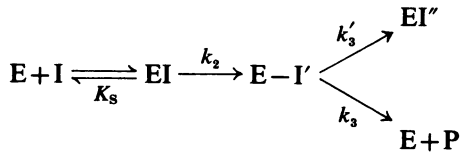
lactamases [23]. The implication from the early work [1,2] was taken to be that the first covalent interaction between the β-lactamase and 6-β-bromopenicillanate, and therefore probably with all substrates, was nucleophilic attack of the serine-70 hydroxy group on the β-lactam carbonyl group. The present results suggest that the thiol β-lactamase employs cysteine-70 analogously.

The conclusions from the spectra described above were further supported by similar experiments with clavulanic acid. The thiol β-lactamase was also irreversibly inactivated by this class A β-lactamase inhibitor, whose interaction with the RTEM-1 enzyme has been carefully explored by Knowles and co-workers [24–26]. One irreversibly inhibited species in the case of the latter enzyme is believed to have the structure (III) (X = O), which, like (II), is a vinylogous carbamoate, and is characterized by an absorption band at 280 nm, $\epsilon = 16000\ M^{-1}\cdot cm^{-1}$ [27]. The isolated complex of thiol RTEM-1 β-lactamase and clavulanate displayed a chromophore at 308 nm ($\epsilon = 17700\ M^{-1}\cdot cm^{-1}$). This result suggests that the analogous structure (III) (X = S) is formed, which, as in the case of 6-β-bromopenicillanate, must arise from nucleophilic attack of the thiol on the β-lactam carbonyl group.



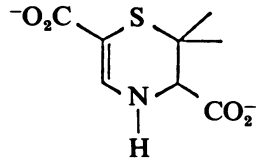
(III)

The kinetics of the reaction between *B. cereus* β-lactamase I and 6-β-bromopenicillanate were accommodated by Scheme 1, where EI is the non-covalent complex of the enzyme with 6-β-bromopenicillanate, E–I′ is the normal acyl-enzyme, EI″ is the inert complex (II), and where the value of n ($n = k_3/k_3'$) was found to be close to zero, i.e. virtually no turnover accompanied inactivation [28]. The same scheme can be applied to the



inactivation of thiol RTEM-1 β-lactamase. Fig. 1 shows that in this case also n is close to zero, i.e. $k_3' \gg k_3$. Kinetic studies, under conditions of 6-β-bromopenicillanate in large excess over enzyme, showed that the inactivation process was pseudo-first-order in nature, where the pseudo-first-order rate constant was linear with 6-β-bromopenicillanate concentration up to 1 mM; thence [28], $K_s \geqslant 1$ mM, $k_2 \geqslant 2.8 \times 10^{-2}\ s^{-1}$ and $k_2/K_s = 28\ s^{-1}\cdot M^{-1}$.

Comparative experiments were performed with the native RTEM-2 enzyme (no significant functional differences have been detected between RTEM-1 and RTEM-2 β-lactamases [29]). This enzyme is also irreversibly inhibited by 6-β-bromopenicillanate, yielding an inhibited enzyme containing the typical chromophore at 323 nm ($\epsilon = 12900\ M^{-1}\cdot cm^{-1}$). A titration with 6-β-bromopenicillanate, analogous to that shown in Fig. 1, yielded a value of n of 105, i.e. $k_3 \geqslant k_3'$. In this case, kinetic studies were made of the rates of appearance of the turnover product (IV) [6-β-bromopenicilloate, the presumed primary product, cyclizes to compound (IV) very rapidly under these conditions, such that the rates of the enzyme-catalysed reaction can be monitored by the appearance of compound (IV) spectrophotometrically at



(IV)

306 nm]. Under conditions of excess 6-β-bromopenicillanate, pseudo-first-order kinetics were again observed, where the pseudo-first-order rate constant was linear with 6-β-bromopenicillanate concentration up to 0.12 mM. Under these circumstances, with the assumptions of $n = 105$ (above) and $k_3 > k_2$ [28], the observed pseudo-first-order rate constant is given by $k_2[I]_0/K_s$, where $[I]_0$ is the initial 6-β-bromopenicillanate concentration. Thence, $K_s \geqslant 0.12$ mM, $k_2 \geqslant 4.9\ s^{-1}$ and $k_2/K_s = 4.1 \times 10^4\ s^{-1}\cdot M^{-1}$.

A comparison between the results obtained with the two enzymes, native and thiol mutant, is instructive. Both are inhibited by 6-β-bromopenicillanate, yielding characteristic covalently bound chromophores, which the above discussion suggests are (II) (X = O) and (II) (X = S) respectively. The 22 nm red-shift on conversion of (II) (X = O) into (II) (X = S) is comparable with the 28 nm shift on analogous conversion of (III) (X = O) into (III) (X = S) in the similar chromophore produced on clavulanate inhibition. This shift could be used perhaps as a readily performed and persuasive test of any putative naturally occurring thiol β-lactamase. Although the 6-β-bromopenicillanate-inactivated native RTEM-2

enzyme does not regain any activity on overnight incubation in 0.1 M-hydroxylamine solution at pH 6.0, the inactivated thiol enzyme does, although quite slowly ($t_{\frac{1}{2}} \simeq 12$ h). This difference presumably reflects the greater susceptibility of (II) (X = S), a thioester, than that of (II) (X = O) to nucleophilic cleavage.

6-β-Bromopenicillanate is a more effective inhibitor of the thiol enzyme, by one measure, in that no turnover accompanies inactivation. Similarly, the extent of turnover accompanying irreversible inhibition of the thiol β-lactamase by clavulanate ($n = 5$) was markedly less than that of the native enzyme ($n = 115$ [24]). On the other hand, however, the acylation rate of the thiol enzyme (k_2/K_s) is over 600 times lower than that of the native. It is likely that the absence of turnover by the thiol enzyme reflects a much slower deacylation (k_3) of this enzyme also, allowing inactivation (k_3') to appear more effective. Cefoxitin, which slowly (0.024 s^{-1}) acylates the RTEM-2 β-lactamase [19], neither is turned over by, nor acylates, the thiol RTEM-1 enzyme. The generally less effective catalysis by the thiol enzyme agrees with the results obtained by Sigal *et al.* [10] with good β-lactamase substrates, and with all previous experience of the serine-to-cysteine mutation [11–13].

We are grateful to Dr. Irving Sigal, Dr. William DeGrado and Dr. Yuk-Ching Tse Dinh for their assistance in our obtaining the pOTBL-containing HB101 strain of *E. coli* K12. Financial support was provided by the National Institutes of Health and a Sigma Xi Grant-in-Aid of Research (to A. K. K.).

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Received 9 March 1987/5 May 1987; accepted 24 June 1987