Biochem. J. (1987) 241, 893–897 (Printed in Great Britain)

Effects of thiol reagents on *Streptomyces* K15 DD-peptidase-catalysed reactions

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The 26000-M_r DD-peptidase of *Streptomyces* K15 binds one equivalent of thiol reagents as 5,5'-dithiobis-(2-nitrobenzoate) or *p*-chloromercuribenzoate (pCMB). Derivatization of the DD-peptidase by pCMB decreases the efficacy of the initial binding of the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-lactate to the enzyme (K), the rate of enzyme acylation by the donor (k_{+2}) and the rate of enzyme deacylation (k_{+3}). However, the value of the k_{+2}/k_{+3} ratio, and therefore the percentage of total enzyme which, at saturating concentrations of the donor, is present as acyl-enzyme at the steady state of the reaction, are not modified. The enzyme's binding sites for pCMB and benzylpenicillin are not mutually exclusive. But, when compared with the native enzyme, the pCMB-derivatized enzyme undergoes acylation by benzylpenicillin with a decreased second-order-rate constant (k_{+2}/K) value and gives rise to a penicilloyl adduct of increased stability. Since the acyl-enzyme mechanism is not annihilated by pCMB derivatization, it is proposed that basically, and like all the other DD-peptidases/penicillin-binding proteins so far characterized, the *Streptomyces* K15 DD-peptidase is an active-site-serine enzyme.

INTRODUCTION

The 26000-M_r DD-peptidase of *Streptomyces* K15 is a membrane-bound enzyme involved in peptide cross-linking during the last stages of wall peptidoglycan synthesis. Purified to 95% homogeneity in the presence of *N*-cetyl-*NNN*-trimethylammonium bromide (Nguyen-Distèche *et al.*, 1982), this enzyme catalyses transfer of the Ac_2-L-Lys-D-alanyl moiety from amide (Ac_2-L-Lys-D-Ala-D-Ala) and ester (Ac_2-L-Lys-D-Ala-D-Lac) carbonyl donors to various acceptors (HY). The general reaction catalysed is:

$$E + D \underset{}{\overset{K}{\rightleftharpoons}} E\cdot D \xrightarrow{k_{+2}} \underset{\text{Leaving group}}{} E\text{-}D^* \xrightarrow{k_{+3}} \underset{HY}{} E + \text{products} \quad (1)$$

where E is the enzyme, D is the carbonyl donor, E·D is the Michaelis complex; E-D* is the acyl (Ac_2-L-Lys-D-alanyl)-enzyme, K is the dissociation constant, and k_{+2} and k_{+3} are the first-order rate constants.

In this transfer reaction the relative acceptor activity of water, the leaving group D-Ala or D-lactate and an amino compound NH_2-R related to the wall peptidoglycan (e.g. Gly-Gly) is: D-lactate $\ll$ 55.5 M-H_2O $\ll$ D-Ala $\ll$ mM-NH_2-R. It follows that, in water, the enzyme hydrolyses the ester donor to completion without any sign of interference by the leaving group, D-lactate. In contrast, the D-alanine that is released from the peptide donor suppresses the acceptor activity of water, performs attack of the acyl enzyme and regenerates the initial substrate, so that little of the donor is consumed. Addition of Gly-Gly, at millimolar concentrations, to the reaction mixture causes complete channelling of both carbonyl donors to the synthesis of Ac_2-L-Lys-D-Ala-Gly-Gly, i.e. the enzyme functions as a strict DD-transpeptidase. In this process, strong experimental evidence suggests that Gly-Gly not only behaves as an alternate nucleophile at the level of the acyl enzyme but, in addition, influences the initial binding of the donor to the enzyme and enhances the efficacy of the ensuing acylation step (Nguyen-Distèche *et al.*, 1986).

β-Lactam compounds (penicillins, cephalosporins, monobactams) function as carbonyl donors, and reaction (1) above applies. However, (i) the leaving group of the enzyme-acylation step (k_{+2}) is not released and remains part of the acyl (penicilloyl, cephalosporoyl, etc.)-enzyme, and (ii) this acyl enzyme is very-long-lived (k_{+3} has a low absolute value) and the enzyme behaves as a penicillin-binding protein.

The values of the kinetic parameters which govern the interaction between the purified *Streptomyces* K15 DD-peptidase and peptide, depsipeptide and β-lactam carbonyl donors have been determined (Nguyen-Distèche *et al.*, 1986). This present paper describes the effect that pCMB exerts on the enzyme acylation and deacylation steps of the catalysed reactions.

MATERIALS AND METHODS

Enzymes and substrates

Purified *Streptomyces* K15 DD-peptidase, *Bacillus cereus* β-lactamase I, Ac_2-L-Lys-D-Ala-D-Ala, Ac_2-L-Lys-D-Ala-D-Lac; [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac (50 mCi/mmol, Gly-Gly, [^{14}C]Gly-Gly (23 mCi/mmol), benzylpenicillin and [^{14}C]benzylpenicillin (54 mCi/mmol) were the compounds used by Nguyen-Distèche *et al.* (1986).

Abbreviations used: D-Lac (in sequences and equations), D-lactate; pCMB, *p*-chloromercuribenzoate; Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid) ('DTNB'); NEM, *N*-ethylmaleimide; Ac, acetyl.

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Thiol reagents

pCMB and Nbs_2 were from Sigma Chemical Co., St. Louis, MO, U.S.A.; NEM was from Fluka, Buchs, Switzerland; iodoacetate was from Merck, Darmstadt, Germany; and [^{14}C]pCMB (15 mCi/mmol) and [^{14}C]Nbs_2 (30 mCi/mmol) from C.E.A., Gif-sur-Yvette, France.

Phosphate/detergent buffer

All the reaction mixtures contained 0.008% cetyltrimethylammonium bromide and, unless otherwise stated, were in 5 mM-phosphate buffer, pH 7.5.

Estimation of [^{14}C]acyl ([^{14}C]Ac_2-L-Lys-D-alanyl or [^{14}C]benzylpenicilloyl)-enzyme

The radioactive acyl-enzyme was stabilized by adding to the reaction mixture an equal volume of denaturing SDS-containing buffer and maintaining the solution for 30 s in a boiling-water bath. After polyacrylamide-slab-gel electrophoresis in the presence of SDS and fluorography of the gel, the amount of [^{14}C]acyl-enzyme was determined by microdensitometry of the fluorogram [for further details, see Nguyen-Distèche *et al.* (1986)].

Reactions with acylic amide and ester carbonyl donors

The catalysed reactions were:

$$Ac_2\text{-L-Lys-D-Ala-D-Lac} + H_2O \rightarrow Ac_2\text{-L-Lys-D-Ala} + \text{D-Lac} \quad (2)$$

$$[^{14}C]Ac_2\text{-L-Lys-D-Ala-D-Lac} + H_2O \rightarrow [^{14}C]Ac_2\text{-L-Lys-D-Ala} + \text{D-Lac} \quad (3)$$

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + \text{Gly-Gly} \rightarrow Ac_2\text{-L-Lys-D-Ala-Gly-Gly} + \text{D-Ala} \quad (4)$$

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + [^{14}C]\text{Gly-Gly} \rightarrow Ac_2\text{-L-Lys-D-Ala-}[^{14}C]\text{Gly-Gly} + \text{D-Ala}) \quad (5)$$

The carbonyl donor was used at a final concentration of 2 mM (reactions 2, 3 and 5) or 8 mM (reaction 4) and the amino acceptor at a final concentration of 1.66 mM. The specific radioactivities of [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac and [^{14}C]Gly-Gly in the final reaction mixtures were 0.8 mCi/mmol and 1.9 mCi/mmol respectively. D-Lactate and D-alanine were estimated by the dehydrogenase and D-amino acid oxidase techniques respectively. [^{14}C]Ac_2-L-Lys-D-Ala and Ac_2-L-Lys-D-Ala-[^{14}C]Gly-Gly were estimated by radioactive measurements after separation of the relevant reaction products by paper electrophoresis at pH 6.5 (60 V/cm) and at pH 5.6 (20 V/cm) respectively [for further details, see Nguyen-Distèche *et al.* (1986)].

The K_m and $k_{cat.}$ values for the hydrolysis of the ester carbonyl donor were derived from Hanes plots ([Ac_2-L-Lys-D-Ala-D-Lac]/v versus [Ac_2-L-Lys-D-Ala-D-Lac]). The K, K_m and k_{+3}/k_{+2} ratio values were derived from plots of $[E]_0/[E\text{-}D^*]_{ss}$ versus $1/[Ac_2\text{-L-Lys-D-Ala-D-Lac}]$ by using the equation:

$$\frac{[E]_0}{[E\text{-}D^*]_{ss}} = 1 + \frac{k_{+3}}{k_{+2}} + \frac{k_{+3}K}{k_{+2}[D]}$$

where $[E]_0$ is total enzyme and $[E\text{-}D^*]_{ss}$ is the amount of acyl ([^{14}C]Ac_2-L-Lys-D-alanyl)-enzyme accumulated at the steady state of the reaction for a given concentration of radioactive ester carbonyl donor. The k_{+2} and k_{+3} values were computed from the known $k_{cat.}$ and k_{+3}/k_{+2}-ratio values.

In the above experiments, $[E]_0$ was estimated by incubating various amounts of enzyme (0.48–3.85 μM) with 0.18 mM-[^{14}C]benzylpenicillin (30 mCi/mmol) for 10 min at 37 °C in 15 μl (final vol.) of phosphate/detergent buffer, under which conditions all of the enzyme was converted into [^{14}C]benzylpenicilloyl-enzyme. After addition of 5 μl of a 0.1 M non-radioactive benzylpenicillin solution, the [^{14}C]benzylpenicilloyl-enzyme was stabilized and estimated as described above. In turn, $[E\text{-}D^*]_{ss}$ was estimated by incubating the enzyme (7.7 μM) for 20 s at 37 °C with various concentrations of [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac [0.5–4.5 mM; 30 mCi/mmol, in 15 μl (final vol.) of phosphate/detergent buffer]. The acyl ([^{14}C]Ac_2-L-Lys-D-alanyl)-enzyme was stabilized and estimated as described above.

Reaction with [^{14}C]benzylpenicillin

Estimation of k_{+3}. An enzyme sample (21 μM) was incubated for 10 min at 37 °C with 0.2 mM-[^{14}C]benzylpenicillin (54 mCi/mmol), in 25 μl of phosphate/detergent buffer, supplemented with an equal volume of precooled acetone, maintained at −20 °C for 1 h, and then centrifuged at 20800 g for 20 min at −10 °C. The pellet (i.e. [^{14}C]benzylpenicilloyl-enzyme) was dissolved in 30 mM-potassium phosphate, pH 7.5, containing 0.05% detergent and the solution was treated for 3 min at 30 °C with amounts of β-lactamase sufficient to destroy any free benzylpenicillin. The rate of release of the [^{14}C]acyl moiety at 37 °C was determined as a function of time as described by Leyh-Bouille *et al.* (1986) and k_{+3} was estimated by using the equation

$$\ln([E\text{-}D^*]_t/[E\text{-}D^*]_0) = -k_{+3}t$$

where $[E\text{-}D^*]_0$ is the preformed [^{14}C]acyl-enzyme and $[E\text{-}D^*]_t$ is the acyl enzyme after time t of incubation.

Estimation of k_{+2}/K. Enzyme samples (1.3 μM) were incubated for 10 min at 37 °C with various concentrations of [^{14}C]benzylpenicillin (from 6 to 300 μM; 54 mCi/mmol) in 30 μl (final vol.) of phosphate/detergent buffer. The reactions were stopped by adding 5 μl of a 0.1 M non-radioactive benzylpenicillin solution and the [^{14}C]acyl-enzyme was stabilized and measured as described above. The second-order rate constant of enzyme acylation (k_{+2}/K) was estimated by using the equation:

$$\frac{-\ln\left(1 - \frac{[E\text{-}D^*]}{E_0}\right)}{t} = \frac{k_{+2}}{K}[D]$$

which is valid for $[D] \ll K$, a condition known to be fulfilled as shown by Leyh-Bouille *et al.* (1986).

Reaction with ^{14}C-labelled thiol reagents: isolation of radioactive adducts

Enzyme samples (18 μM) were incubated at 37 °C for 30 min (i) with [^{14}C]pCMB (from 30 to 130 μM; 15 mCi/mmol) in 10 mM-$NaHCO_3$ containing 0.008% detergent; or (ii) with [^{14}C]Nbs_2 (2 mM; 30 mCi/mmol) in 25 mM-potassium phosphate, pH 8, containing 0.008% detergent. The final volumes were 12 μl. The adducts formed were estimated by radioactivity measurements after chromatography on thin-layer Polygram Sil G (Macherey and Nagel Co., Düren, Germany) in butan-1-ol/acetic acid/pyridine/water (15:4:10:12, by vol.), under which conditions the adducts remained at the

Table 1. Kinetic constants for the interactions between the native and pCMB-derivatized *Streptomyces* K15 DD-peptidase (E) and the ester Ac_2-L-Lys-D-Ala-D-Lac and benzylpenicillin carbonyl donors (D)

The parameters given pertain to the reaction: $E + D \underset{}{\overset{K}{\rightleftharpoons}} E \cdot D \xrightarrow{k_{+2}}$ acyl-enzyme $\xrightarrow{k_{+3}} E +$ products

	D = Ac_2-L-Lys-D-Ala-D-Lac									D = benzylpenicillin	
	Hanes plot		Acyl-enzyme trapping (Fig. 1)								
	$k_{cat.}$ (s^{-1})	K_m (mM)	K_m (mM)	K (mM)	k_{+2} (s^{-1})	k_{+3} (s^{-1})	k_{+3}/k_{+2}	k_{+2}/K ($M^{-1} \cdot s^{-1}$)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	k_{+2}/K ($M^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})
Native	0.5	0.8	1.3	2.2	0.86	1.2	1.4	390	625	155	1×10^{-4}
pCMB-derivatized	0.066	4.2	3.0	5.2	0.11	0.16	1.38	20	16	60	0.3×10^{-4}

origin of the chromatograms, well separated from the excess reagents. In some cases, the adducts were precipitated by cold acetone as described above for [^{14}C]benzylpenicilloyl-enzyme.

RESULTS

Binding of thiol reagents to *Streptomyces* K15 DD-peptidase

Incubation of the DD-peptidase (1.7 μM) for 30 min at 37 °C with NEM [2 mM in 15 mM-potassium phosphate (pH 7.5)/0.008% detergent], Nbs_2 [1 mM in 25 mM-potassium phosphate (pH 8)/0.008% detergent] and pCMB (10 μM in 10 mM-$NaHCO_3$/0.008% detergent) inhibited by 90% both the DD-carboxypeptidase and DD-transpeptidase activities of the enzyme. No more than 90% inhibition of the enzyme could be achieved, irrespective of the pCMB concentration used (up to 0.3 mM). Iodoacetate [2 mM in 8 mM-potassium phosphate (pH 7.5)/0.008% detergent] had no effect.

Enzyme samples (10 μM) maximally (i.e. 90%) inhibited by [^{14}C]pCMB or [^{14}C]Nbs_2 were submitted to t.l.c. Radioactivity measurements showed that each reagent bound to the 26000-M_r protein in a 1:1 molar ratio (actual results were 0.82 mol of pCMB and 0.98 mol of Nbs_2/mol of protein). The presence of 3 M-guanidinium chloride in the reaction mixtures did not increase the amount of bound radioactivity, suggesting that the DD-peptidase had one single thiol group.

In order to further characterize the adducts formed, samples of [^{14}C]Nbs_2- or [^{14}C]pCMB-derivatized enzyme were precipitated with cold acetone to remove the excess of reagent and the adducts were redissolved in 30 mM-potassium phosphate, pH 7.5, containing detergent (final protein concn. 18 μM). The following observations were made: (i) virtually no release of radioactivity and no recovery of enzyme activity occurred during a 180 min incubation at 37 °C, showing that the adducts were essentially hydrolytically inert; (ii) addition of 20 mM-dithiothreitol or 20 mM-mercaptoethanol to the derivatized enzyme samples and incubation for 20 min at 37 °C caused the release of 98–99% of the radioactivity (as shown by t.l.c.), but failed to regenerate an active enzyme (though none of these compounds at the aforementioned concentration had any inhibitory effect on the native enzyme); (iii) after treatment with dithiothreitol or mercaptoethanol, complete recovery of enzyme activity could be achieved by precipitation of the protein with cold acetone and redissolution in 30 mM-potassium phosphate, pH 7.5, containing the detergent; (iv) the [^{14}C]pCMB-derivatized protein bound [^{14}C]benzylpenicillin (0.2 mM final concn.; 15 mCi/mmol; 15 min at 37 °C). As shown by t.l.c. and radioactivity measurements, the resulting preparation contained 1.7 equiv. of ^{14}C-labelled compound (i.e. pCMB + benzylpenicillin)/mol of protein. Identical results were obtained when the enzyme was first [^{14}C]benzylpenicilloylated and then [^{14}C]pCMB-derivatized. Hence

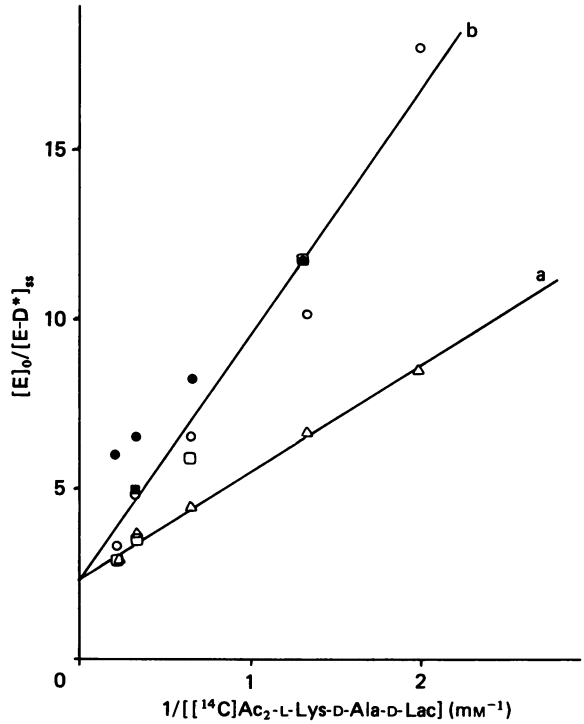

Fig. 1. Plots of $[E]_0/[E\text{-}D^*]_{ss}$ versus 1/[^{14}C]Ac_2-L-Lys-D-Lac for the native (a) and pCMB-derivatized (b) *Streptomyces* K15 DD-peptidase

$[E]_0$ represents total enzyme; $[E\text{-}D^*]_{ss}$ is acyl ([^{14}C]Ac_2-L-Lys-D-alanyl)-enzyme trapped at the steady state of the reaction. a, Data from Nguyen-Distèche *et al.* (1986) (△); b, data from several experiments (□, ○, ●, ■). The correlation coefficient is 0.96.

the DD-peptidase had distinct, non-exclusive, benzylpenicillin- and pCMB-binding sites.

Effects of pCMB on the kinetic parameters of *Streptomyces* K15 DD-peptidase-catalysed reactions

Hydrolysis of Ac_2-L-Lys-D-Ala-D-Lac. The pCMB-derivatized DD-peptidase had low, but detectable, residual DD-carboxypeptidase and DD-transpeptidase activities. The values of the kinetic parameters which govern hydrolysis of Ac_2-L-Lys-D-Ala-D-Lac ($k_{cat.}$, K_m, K, k_{+2}, k_{+3} and k_{+2}/k_{+3}) by both the native and pCMB-derivatized enzymes were determined from Hanes plots and by measuring the amounts of acyl ([^{14}C]Ac_2-L-Lys-D-alanyl)-enzyme accumulated at the steady state of the reaction for various concentrations of the carbonyl donor (Fig. 1). As shown in Table 1, pCMB-derivatization of the DD-peptidase caused a 2.5-fold increase K value, 8-fold-decreased k_{+2} and k_{+3} values and a 20–40-fold-decreased second-order-rate-constant value for enzyme acylation (i.e. k_{+2}/K or $k_{cat.}/K_m$). pCMB, however, did not alter the k_{+2}/k_{+3} ratio value and did not modify the percentage of total enzyme (40%) that would occur as acyl-enzyme at the steady state of the reaction under saturating concentration of the ester carbonyl donor (i.e. for [D] = ∞). Hence the residual carboxypeptidase and transpeptidase activities observed after pCMB treatment of the DD-peptidase were not due to the presence of a contaminating pCMB-insensitive DD-peptidase in the enzyme preparation nor to an incomplete extent of protein derivatization. It actually expressed the low catalytic efficiency of the modified DD-peptidase.

Inactivation by penicillin. The doubly pCMB/[^{14}C]-benzylpenicillin- or Nbs_2/[^{14}C]benzylpenicillin-derivatized DD-peptidase was precipitated with cold acetone and redissolved in 30 mM-potassium phosphate, pH 7.5, containing detergent (final concn. of the double adducts 21 μM). Release of the radioactivity at 37 °C as a function of time proceeded with a k_{+3} rate value of 0.3×10^{-4} s^{-1} or 0.24×10^{-4} s^{-1} respectively. In turn, [^{14}C]benzylpenicilloylation of the pCMB-derivatized DD-peptidase proceeded with a second-order rate constant of 60 M$^{-1} \cdot$ s^{-1}. By reference to the native enzyme (Table 1), pCMB binding decreased both the efficacy of protein acylation by benzylpenicillin (k_{+2}/K) by a factor of 2.5 and the rate of breakdown of the bound benzylpenicilloyl moiety (k_{+3}) by a factor of 3.4.

DISCUSSION

The *Streptomyces* K15 26000-M_r DD-peptidase binds 1 mol of pCMB (and other thiol reagents)/mol of protein to form, by derivatization of an enzyme's thiol group, a stable adduct with a low enzymic activity. Breakdown of the adduct by mercaptoethanol treatment yields a protein of low catalytic efficacy but which regains full enzymic activity after precipitation with acetone (suggesting that protein unfolding and refolding takes place). The thiol-reagent-binding site in the native enzyme is readily accessible to pCMB. Although the protein possesses two or three cysteine residues (B. Joris, M. Nguyen-Distèche & M. Leyh-Bouille, unpublished work), unfolding of the protein by treatment with 3 M-guanidinium chloride does not cause exposure of any additional thiol group(s). The search for the possible presence of disulphide bridges failed (M. Leyh-Bouille & M. Nguyen-Distèche, unpublished work), suggesting that the pCMB-insensitive thiol group(s) present in the enzyme might be blocked [for example, in the form of a 'cysteinyl acylglycerol thioether' as it occurs in the lipoprotein of *Escherichia coli* (Hantke & Braun, 1973) or in the membrane-bound β-lactamase of *Bacillus licheniformis* (Lai *et al.*, 1981; Nielsen *et al.*, 1981)].

pCMB derivatization decreases the efficacy of the initial binding of the ester donor Ac_2-L-Lys-D-Ala-D-Lac to the enzyme (K), the rate of enzyme acylation by the donor (k_{+2}) and the rate of enzyme deacylation (k_{+3}). However, at a saturating concentration of the donor, pCMB binding does not affect the proportion of total enzyme which is present as acyl (Ac_2-L-Lys-D-alanyl)-enzyme at the steady state of the reaction (unmodified k_{+2}/k_{+3} value).

The enzyme's pCMB- and benzylpenicillin-binding sites are not mutually exclusive, but pCMB derivatization also causes both a decrease of the second-order rate constant of enzyme acylation by benzylpenicillin (k_{+2}/K) and a decrease of the rate of enzyme deacylation (k_{+3}).

From the foregoing it can be concluded that, central to the acyl-enzyme mechanism through which the *Streptomyces* K15 DD-peptidase operates on acyclic and β-lactam carbonyl donors, is the activation of an enzyme's nucleophile other than the pCMB-binding site, presumably a serine residue, since it occurs in all the penicillin-binding DD-peptidases so far characterized.

The *Streptomyces* K15 DD-peptidase behaves as a pCMB-sensitive, active-site-serine enzyme such as carboxypeptidase Y (Bai & Hayashi, 1979; Breddam, 1983), thermitase from *Thermoactinomyces vulgaris* (Stepanov, 1980) and, among the penicillin-recognizing enzymes, the *Escherichia coli* DD-peptidase/penicillin-binding protein no. 5 [where a cysteine residue occurs at position 71 on the carboxy side of the active-site serine residue (Broome-Smith & Spratt, 1984)] and the β-lactamase of *Klebsiella aerogenes* (Ezard *et al.*, 1986). Curtis & Strominger (1978) first proposed that pCMB binding to the *E. coli* DD-peptidase did not prevent acyl-enzyme formation, but blocked the enzyme-deacylation step. More recently, Broome-Smith & Spratt (1984) showed that both steps were affected. The Nbs_2-sensitive cysteine residue in the *K. aerogenes* β-lactamase is not accessible to the reagent unless the protein is denatured by guanidinium chloride treatment. The Nbs_2-derivatized and refolded β-lactamase hydrolyses benzylpenicillin with a 24-fold-decreased $k_{cat.}$ value and a 3-fold increased K_m value. It remains readily inactivable by 6β-iodopenicillanate, and the β-iodopenicillanate-derivatized β-lactamase binds Nbs_2.

This work was supported in part by the Fonds de la Recherche Scientifique Médicale, Brussels, Belgium (Contract no. 3.4507.83), an Action Concertée with the Belgian Government (Convention 79/84-I1) and a Convention Tripartite between the Région wallonne, Continental Pharma and the University of Liège.

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Received 27 October 1986; accepted 14 November 1986