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Active-site-serine D-alanyl-D-alanine-cleaving-peptidase-catalysed acyl-transfer reactions

Procedures for studying the penicillin-binding proteins of bacterial plasma membranes

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Under certain conditions, the values of the parameters that govern the interactions between the active-site-serine D-alanyl-D-alanine-cleaving peptidases and both carbonyl-donor substrates and β-lactam suicide substrates can be determined on the basis of the amounts of (serine ester-linked) acyl-protein formed during the reactions. Expressing the 'affinity' of a β-lactam compound for a DD-peptidase in terms of second-order rate constant of enzyme acylation and first-order rate constant of acyl-enzyme breakdown rests upon specific features of the interaction (at a given temperature) and permits study of structure–activity relationships, analysis of the mechanism of intrinsic resistance and use of a 'specificity index' to define the capacity of a β-lactam compound of discriminating between various sensitive enzymes. From knowledge of the first-order rate constant of acyl-enzyme breakdown and the given time of incubation, the β-lactam compound concentrations that are necessary to achieve given extents of DD-peptidase inactivation can be converted into the second-order rate constant of enzyme acylation. The principles thus developed can be applied to the study of the multiple penicillin-binding proteins that occur in the plasma membranes of bacteria.

INTRODUCTION

The active-site-serine D-alanyl-D-alanine-cleaving peptidases are bacterial enzymes that exhibit a unique optical specificity (Ghuysen *et al.*, 1984; Frère & Joris, 1985): the scissile peptide bond extends between two D-alanine residues in α-position to a free carboxylate group. These enzymes also function as esterases on D-Ala-D-lactate-terminated (and other) depsipeptides (Yocum *et al.*, 1982; Pratt *et al.*, 1985). In those cases where the reaction pathway has been elucidated, the catalysed acyl transfer proceeds via the formation of a serine ester-linked acyl-enzyme (Frère *et al.*, 1976).

The active-site-serine DD-peptidases are targets specifically attacked by the β-lactam antibiotics (penicillins, cephalosporins, monobactams). β-Lactam compounds are recognized as carbonyl-donor analogues but the reaction flux stops, at least for a long time, at the level of the corresponding acyl-enzyme, thus conferring on the active-site-serine DD-peptidases the property of behaving as penicillin-binding proteins (PBPs) (Spratt, 1983; Ghuysen *et al.*, 1984; Frère & Joris, 1985).

All bacteria examined thus far possess, bound to their plasma membrane, multiple PBPs that are involved in the assembly and constant remodelling of the bacterial wall peptidoglycan (Spratt, 1983). In several instances these PBPs have been shown to possess DD-peptidase (carboxypeptidase and/or transpeptidase) activities and penicillin binding has been shown to occur by acylation of a serine residue (Frère *et al.*, 1976; Keck *et al.*, 1985; Broome-Smith *et al.*, 1985). Currently, the 'affinity' of a PBP for a β-lactam compound (i.e. the inactivating potency of a β-lactam compound towards a PBP) is defined as the concentration (often expressed in μg/ml) required to achieve 50% saturation of the PBP after a given time (5 to 60 min have been used) at a specified temperature (often 37 °C). Although this definition has been very widely used, it does not permit comparison of data obtained under differing conditions and does not permit study of the interaction between PBPs and β-lactam compounds in terms of structure–activity relationships.

The discussion presented below shows that, if acyl-enzyme breakdown is not much faster than acyl-enzyme formation, the amount of acyl-enzyme that is formed during reaction between the active-site-serine DD-peptidases and peptide or depsipeptide substrates can be used to calculate the values of the parameters that govern the interaction. On the basis of the equations thus developed for substrates, this paper also presents, in some detail, the various factors that must be considered in order to define, in terms of the kinetic parameters involved, the efficacy with which the β-lactam compounds inactivate the active-site-serine DD-peptidases or immobilize the PBPs in a biologically inactive form. Some of the equations derived in the present paper might exhibit similarities to those derived by Morrison (1982) for slow-binding inhibitors. However, the analysed phenomena are different, since the β-lactam compounds are irreversibly transformed by the DD-peptidases (PBPs), in contrast with the inhibitors considered by Morrison (1982). With regard to the mechanistic properties of the DD-peptidases (and PBPs), the present discussion is based, at least in concept, on previous work dealing with the acyl-transfer reactions catalysed by the well-

Abbreviation used: PBP, penicillin-binding protein.

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known active-site-serine peptidases such as chymotrypsin (Fersht, 1985) and the R61 and R39 DD-peptidases (Frère *et al.*, 1975*a*; Fuad *et al.*, 1976).

THEORY

Kinetics of R-D-Ala-D-Ala or R-D-Ala-D-lactate carbonyl-donor substrates

General reaction and basic equations. The active-site-serine DD-peptidases (E) catalyse transfer of the electrophilic group R-D-alanyl from amide (R-D-Ala-D-Ala-OH) and ester (R-D-Ala-D-lactate) carbonyl-donor substrates (D) to a nucleophilic acceptor (HY), via formation of an acyl (R-D-alanyl)-enzyme (E–D*). With the definitions E·D = Michaelis complex, K = dissociation constant of E·D, k_{+2} and k_{+3} = first-order rate constants, P_1 = leaving group (D-Ala or D-Lac) and P_2 = second reaction product (R-D-Ala-CO-Y), the general reaction catalysed is:

$$E+D \overset{K}{\rightleftharpoons} E\cdot D \xrightarrow[P_1]{k_{+2}} E\text{–}D^* \xrightarrow[HY]{k_{+3}} E+P_2 \qquad (1)$$

with [D] ≫ [E] and assuming that E·D is in rapid equilibrium with free enzyme and free carbonyl-donor substrate).

The reaction obeys the following equations:

$$k_a = \frac{k_{+2}}{1+\frac{K}{[D]}} \quad \text{or} \quad \frac{1}{k_a} = \frac{1}{k_{+2}} + \frac{K}{k_{+2}[D]} \qquad (2)$$

which for [D] ≪ K simplifies to:

$$k_a = \frac{k_{+2}[D]}{K} \qquad (3)$$

$$k_{cat.} = \frac{k_{+2}k_{+3}}{k_{+2}+k_{+3}} \qquad (4)$$

$$K_m = \frac{Kk_{+3}}{k_{+2}+k_{+3}} \qquad (5)$$

$$\frac{k_{cat.}}{K_m} = \frac{k_{+2}}{K} \qquad (6)$$

with k_a = pseudo-first-order rate constant of E–D* formation at a given [D] value, $k_{cat.}/K_m$ = catalytic efficiency at low carbonyl-donor substrate concentrations (≤ 0.1 K_m) and k_{+2}/K = second-order rate constant of enzyme acylation by the carbonyl-donor substrate.

When, at a given [D], the system reaches the steady state, the concentrations of E, E·D and E–D* remain stable and the ratio of total enzyme $[E]_0$ to acyl-enzyme $[E\text{–}D^*]_{ss}$ is:

$$\frac{[E]_0}{[E\text{–}D^*]_{ss}} = \frac{k_a+k_{+3}}{k_a} = 1+\frac{k_{+3}}{k_{+2}}+\frac{Kk_{+3}}{k_{+2}[D]} \qquad (7)$$

Fig. 1 shows how $[E\text{–}D^*]_{ss}/[E]_0$ varies as a function of increasing values of k_{+2}/k_{+3} (from 0.01 to 100) for various [D] values such that [D]/K ranges from ∞ to 0.01.

Finally, at a given [D], the time necessary for the acyl-enzyme to reach a certain percentage of its steady-state concentration is:

$$t = \frac{-\ln\left(1-\frac{[E\text{–}D^*]}{[E\text{–}D^*]_{ss}}\right)}{k_{+3}+k_a} \qquad (8)$$

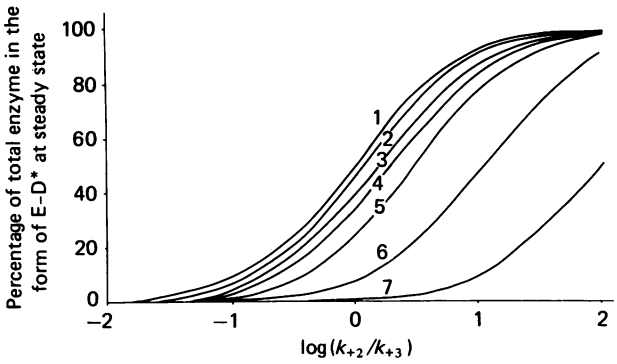


Fig. 1. Accumulation of acyl-enzyme E–D* at the steady state of a catalysed three-step reaction:

$$E+D \overset{K}{\rightleftharpoons} E\cdot D \xrightarrow{k_{+2}} E\text{–}D^* \xrightarrow{k_{+3}} E+P$$

Percentage of total enzyme in the form of E–D* at the steady state is plotted as a function of k_{+2}/k_{+3} for various values of [D]/K: ∞ (curve 1), 10 (curve 2), 2 (curve 3), 1 (curve 4), 0.5 (curve 5), 0.1 (curve 6) and 0.01 (curve 7).

For example, for $k_{+2} = k_{+3} = 10\ s^{-1}$, K = 1 mM and [D] = 10 mM, 47% of total enzyme occurs as acyl-enzyme at the steady state of the reaction (eqn. 7) and 99% of the steady-state value is reached after 0.24 s (eqn. 8).

Practical applications. The plot $[E]_0/[E\text{–}D^*]_{ss}$ versus 1/[D] (eqn. 7) is a straight line from which the values of K, K_m and k_{+3}/k_{+2} can be derived. The slope is equal to Kk_{+3}/k_{+2}, the intercept on the abscissa is equal to $-1/K_m$, the intercept on the ordinate is equal to $1+k_{+3}/k_{+2}$, and, at [D] = ∞, the maximal concentration of acyl-enzyme at the steady state of the reaction is $[E\text{–}D^*]_{ss}/[E]_0 = k_{+2}/(k_{+2}+k_{+3})$. Hence k_{+3}/k_{+2} and K are data experimentally available from acyl-enzyme measurements (with the use of a radioactive carbonyl donor such that, once P_1 has diffused away, a radioactive E–D* is produced). From knowledge of k_{+3}/k_{+2} and use of the $k_{cat.}$ value (as derived from Hanes plots), k_{+2} and k_{+3} can be calculated from eqn. (4):

$$k_{+2} = k_{cat.}+k_{cat.}\cdot\left(\frac{k_{+2}}{k_{+3}}\right) \qquad (9)$$

$$k_{+3} = k_{cat.}+k_{cat.}\cdot\left(\frac{k_{+3}}{k_{+2}}\right) \qquad (10)$$

For $k_{+3} \gg k_{+2}$ (rate-determining formation and low steady-state proportion of acyl-enzyme), $k_{cat.} = k_{+2}$ and $K_m = K$ (Hess, 1971). For $k_{+3} \ll k_{+2}$ (rate-determining breakdown and large steady-state proportion of acyl-enzyme), $k_{cat.} = k_{+3}$ and $K_m = Kk_{+3}/k_{+2}$, eqn. (7) becomes:

$$\frac{[E]_0}{[E\text{–}D^*]_{ss}} = 1+\frac{Kk_{+3}}{k_{+2}[D]} = 1+\frac{K_m}{[D]} \qquad (11)$$

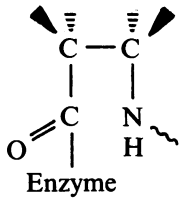
The ratio k_{+2}/K can be computed but the individual values of k_{+2} and K remain undetermined. Eqn. (11) shows that large accumulation of acyl-enzyme at the steady state of the reaction may occur even at [D] ≪ K (see curve 7 in Fig. 1). With knowledge of K_m and [D], eqn. (11) gives

the maximal amount of enzyme that can be trapped as acyl-enzyme (i.e. for $k_{+3} \ll k_{+2}$).

Kinetics of β-lactam carbonyl-donor suicide substrates

General reaction. Upon reaction with β-lactam compounds, the active-site-serine DD-peptidases become immobilized in the form of acyl-enzyme. Constant k_{+3} has a small absolute value.

As a consequence of the endocyclic nature of the scissile amide bond in the β-lactam ring, the leaving group P_1 remains part of the acyl-enzyme and does not diffuse away from the enzyme active site:



Hence reaction (1) becomes:

$$E+D \overset{K}{\rightleftharpoons} E\cdot D \xrightarrow{k_{+2}} E\text{–}D^* \xrightarrow[HY]{k_{+3}} E+P \qquad (12)$$

and the active-site-serine DD-peptidases behave as penicillin-binding proteins. For large β-lactam compound concentrations, formation of E–D* can be very rapid and $[E\text{–}D^*]_{ss}$ can be very close to $[E]_0$. For example, for $K = 1$ mM, $k_{+2} = 10\ s^{-1}$, $k_{+3} = 1 \times 10^{-4} s^{-1}$ and with $[D] = 10$ mM, the enzyme catalytic centre 'turns over' every 166 min ($k_{cat.} = k_{+3}$), the acyl-enzyme at the steady state of the reaction is 99.99% of total enzyme (eqn. 11), and 99% of the steady-state value is reached after 0.5 s {eqn. (8) with $k_a = k_{+2}/(1+K/[D])$; see eqn. (2)}.

When, in reaction (12), D is benzylpenicillin and HY is H_2O, the expected reaction product P is benzylpenicilloate. Active-site-serine DD-peptidases function as β-lactamases of very poor efficacy (low k_{+3} values) (Frère *et al.*, 1974). In some cases, however, the reaction is a branched pathway. The benzylpenicilloyl-enzyme slowly undergoes both direct hydrolysis into benzylpenicilloate and fragmentation with generation of phenylacetylglycyl-enzyme. Once formed, this latter acyl-enzyme undergoes hydrolysis with release of phenylacetylglycine. Since, in this process, fragmentation is rate-determining (Frère *et al.*, 1975*b*, 1978), k_{+3} refers to this particular acyl-enzyme rearrangement.

If the reaction mixture contains an amino compound H_2N–X known to be an effective acceptor in transfer reactions involving carbonyl-donor substrates, partitioning between H_2O and H_2N–X occurs only at the level of phenylacetylglycyl-enzyme (Marquet *et al.*, 1979). The inertness of benzylpenicilloyl-enzyme strongly suggests that the benzylpenicilloyl moiety prevents access to or functioning of the enzyme amino-acceptor site. Note, however, that a simple amino nucleophile, such as hydroxylamine, attacks the benzylpenicilloyl-enzyme.

The equations developed for the amide and ester carbonyl-donor substrates (with $k_{+3} \ll k_{+2}$) apply to β-lactam suicide substrates. But, because k_{+3} has (normally) a very small absolute value, the interaction between the active-site-serine DD-peptidases and β-lactam compounds is peculiar in several respects.

Acyl-enzyme breakdown: calculation of k_{+3}. Calculation of k_{+3} rests upon the equation:

$$\ln\left(\frac{[E\text{–}D^*]_t}{[E\text{–}D^*]_0}\right) = -k_{+3}t \qquad (13)$$

where $[E\text{–}D^*]_0$ = preformed acyl-enzyme and $[E\text{–}D^*]_t$ = acyl-enzyme after time t of incubation in a medium deprived of free β-lactam compound (Frère *et al.*, 1974). With knowledge of k_{+3}, the half-life of the acyl-enzyme is given by:

$$\text{Half-life} = \frac{-\ln 0.5}{k_{+3}} = \frac{0.69}{k_{+3}} \qquad (14)$$

Practical procedures for determining k_{+3}. Determination of k_{+3} rests upon eqn. (13). It involves measurements, as a function of time, of the release of the acyl moiety (from an acyl-enzyme obtained with a radioactive β-lactam compound) or the recovery of the enzyme activity. In this latter case, samples of acyl-enzyme removed after increasing times of incubation are treated, in a second step, with a carbonyl-donor substrate. The time of incubation with the substrate must be short when compared with the half-life of the acyl-enzyme (since the acyl-enzyme continues to decay during that incubation) or a suitable correction must be utilized (Frère *et al.*, 1974). Alternatively, the acyl-enzyme and the substrate are mixed together and the amount of product $[P]_t$ formed from the substrate on subsequent incubation (Frieden, 1970) is given by:

$$[P]_t = v_f\left(t - \frac{1}{k_{+3}}(1-e^{-k_{+3}t})\right) \qquad (15)$$

where v_f is the rate of product formation by the fully re-activated enzyme. The value of $1/k_{+3}$ is computed by extrapolating on the abscissa, the linear portion of the curve obtained for large values of t {under which condition $[P]_t = v_f(t - 1/k_{+3})$}. This method is especially suitable (Marquet *et al.*, 1974) when the acyl-enzyme does not have a very long half-life or the free enzyme has a low enzyme activity.

Acyl-enzyme formation: calculation of the k_a values. Eqn. (8) can be used only if $[E\text{–}D^*]_{ss}$ is measured. Most often, however, $[E\text{–}D^*]_{ss}$ is not known except at those β-lactam concentrations that cause complete acylation of the enzyme, under which conditions $[E\text{–}D^*]_{ss} = [E]_0$.

In contrast, the $[E\text{–}D^*]/[E]_0$ ratio values are experimentally available data and are related to k_a by:

$$\frac{[E\text{–}D^*]}{[E]_0} = \frac{k_a}{k_{+3}+k_a}\cdot(1-e^{-(k_{+3}+k_a)t}) \qquad (16)$$

which, for $k_a \gg k_{+3}$, simplifies to

$$-\ln\left(1-\frac{[E\text{–}D^*]}{[E]_0}\right) = k_a t \qquad (17)$$

Eqn. (16) is general. Use of eqn. (17) is justified if the time of incubation is much shorter than the half-life of the acyl-enzyme and if the β-lactam compound, at the concentration used, is able to cause complete acylation of the enzyme at the steady state of the reaction {indeed, the condition $k_a \gg k_{+3}$ implies $[E\text{–}D^*]_{ss}/[E]_0 = 1$; see eqn. (7)}.

The use of eqns. (16) and (17) is illustrated by the saturation curves shown in Fig. 2. These curves were constructed with β-lactam compound concentrations

Table 1. Determination of the pseudo-first-order rate constant (k_a) of enzyme acylation by increasing β-lactam compound concentrations [D] and the second-order rate constant (k_{+2}/K) of enzyme acylation, by using eqn. (21) and the experimental points of curve 2 ($t = 900$ s) of Fig. 2

For each β-lactam concentration, the starting k_a value for the successive approximations is chosen by assuming a steady-state situation {$[E\text{–}D^*]_{ss}/[E]_0 = k_a/(k_{+3}+k_a)$; see eqn. (7)}.

[D] (μM)	$\left(\frac{E\text{–}D^*}{E_0}\right)_{obs.}$	Approx. k_a (s^{-1})	$\left(\frac{E\text{–}D^*}{E_0}\right)_{calc.}$ on basis of approx. k_a	$k_a/[D]$† ($M^{-1}\cdot s^{-1}$)
0.05	0.24	0.3×10^{-3}	0.16	
		0.45×10^{-3}	0.226	
		0.5×10^{-3}	0.24	10000
0.10	0.42	0.7×10^{-3}	0.32	
		0.9×10^{-3}	0.39	
		1.0×10^{-3}	0.42	10000
0.15	0.54	1.2×10^{-3}	0.46	
		1.4×10^{-3}	0.51	
		1.5×10^{-3}	0.54	10000
0.20	0.62	1.6×10^{-3}	0.56	
		1.8×10^{-3}	0.59	
		2.0×10^{-3}	0.62	10000
0.30	0.73	2.7×10^{-3}	0.70	
		3.0×10^{-3}	0.73	10000
0.4	0.79	3.8×10^{-3}	0.78	
		4.0×10^{-3}	0.79	10000

† The fact that k_a remains proportional to [D] (the values of $k_a/[D]$ remain constant) indicates that $[D] \ll K$ and $k_a/[D] = k_{+2}/K$.

ranging from 10 nM to 10 μM and incubation times of 50, 200, 900 and 4000 s, and assuming a K value of 1 mM, a k_{+2} value of 10 s^{-1} (i.e. a k_{+2}/K value of 10000 $M^{-1}.s^{-1}$) and a k_{+3} value of 1×10^{-3} s^{-1} (the acyl-enzyme is supposed to have a half-life of 690 s). To construct these curves, the extents of acylation of the enzyme at the steady state were calculated for each β-lactam compound concentration on the basis of eqn. (11), and from the $[E\text{–}D^*]_{ss}$ values thus obtained the extents of saturation of the PBP after various times of incubation were calculated on the basis of eqn. (8). For incubation times longer than 4000 s, the steady state is reached and the saturation curves superimpose that obtained at $t = 4000$ s.

Assume that the saturation curves of Fig. 2 are experimental data and that the half-life of the acyl-enzyme is (erroneously) supposed to be much larger than the incubation times used. At [D] = 0.4 μM the theoretical k_a value is $4\times10^{-3}s^{-1}$ (see eqn. 2), and eqn. (17) yields k_a values of 3.96×10^{-3}, 3.45×10^{-3}, 1.68×10^{-3}, and 0.39×10^{-3} s^{-1} at t values of 50, 200, 900 and 4000 s respectively. For $t > 4000$ s the corresponding k_a values decrease proportionally to $1/t$, since $[E\text{–}D^*]/[E]_0$ remains unchanged. Obviously, a correct k_a value (i.e. $4\times10^{-3}s^{-1}$) can be computed only from the data obtained at the lowest t value (which is 14-fold smaller than the half-life of the acyl-enzyme). Conversely, irrespective of the incubation time, the value of k_a can be estimated correctly

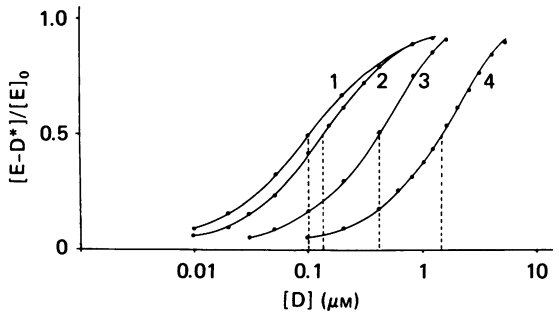


Fig. 2. Saturation (acylation) of a PBP by increasing β-lactam concentrations with incubation times of 4000 s (curve 1), 900 s (curve 2), 200 s (curve 3) and 50 s (curve 4)

$K = 1$ mM, $k_{+2} = 10$ s^{-1} and $k_{+3} = 1\times10^{-3}$ s^{-1} in all cases.

from eqn. (16). As shown in Table 1, in the case of curve 2 ($t = 900$ s) the procedure involves successive approximations. Various k_a values are introduced in eqn. (16) until the $[E\text{–}D^*]/[E]_0$ ratio values thus calculated are equal to the experimentally determined $[E\text{–}D^*]/[E]_0$ ratio values.

Finally, eqn. (7) may have practical usefulness for the determination of k_a at least in those particular cases where k_{+3} is not much smaller than k_a and an appreciable proportion of enzyme occurs in the active form at the steady state of the reaction. If $[D] \ll K$, under which conditions $[E\cdot D] \simeq 0$ and $[E]_{ss}/[E]_0 = k_{+3}/(k_a+k_{+3})$ (see eqn. 7), and if k_{+3} is known, then k_a can be computed by measuring the residual enzyme activity at the steady state (L. Varetto, D. Cesa, J.-M. Frère, J.-M. Ghuysen & C. Houssier, unpublished work).

Determination of the values of the second-order rate constant k_{+2}/K and the individual constants K and k_{+2} of enzyme acylation. From the k_a values measured at various β-lactam compound concentrations, the parameters of enzyme acylation can be determined graphically. (i) If the plot of k_a versus [D] is a straight line, the condition $[D] \ll K$ is fulfilled (see eqn. 3) and the slope of the line is equal to k_{+2}/K. (ii) If the plot of k_a versus [D] shows deviation from linearity, the condition $[D] \ll K$ is not fulfilled (see eqn. 2). Under these conditions, the reciprocal plot of $1/k_a$ versus $1/[D]$ is a straight line, the intercept on the ordinate gives $1/k_{+2}$ and the slope is equal to K/k_{+2}, or, alternatively, the plot of $[D]/k_a$ versus [D] is a straight line, the intercept on the ordinate is K/k_{+2} and the slope is equal to $1/k_{+2}$ (thus permitting calculation of the individual values of K and k_{+2}).

Combining eqn. (17) (which is valid for $k_{+3} \ll k_a$) with eqn. (3) or eqn. (2) gives respectively:

$$\frac{-\ln\left(1-\frac{[E\text{–}D^*]}{[E]_0}\right)}{t} = k_a = \frac{k_{+2}[D]}{K} \quad (\text{if } [D] \ll K) \quad (18)$$

and

$$-\frac{t}{\ln\left(1-\frac{[E\text{–}D^*]}{[E]_0}\right)} = \frac{1}{k_a} = \frac{1}{k_{+2}} + \frac{K}{k_{+2}[D]} \quad (\text{if } [D] \simeq K) \quad (19)$$

Eqn. (18) expresses, for a given k_{+2}/K value, the extent of enzyme acylation as a function of the β-lactam compound concentration and the incubation time used. Hence 99% or 50% of enzyme acylation is achieved when the product of the β-lactam compound concentration ($[D]_{0.99}$ or $[D]_{0.5}$) multiplied by the incubation time ($t_{0.99}$ or $t_{0.5}$) is such that:

$$([D]t)_{0.99} = -\ln 0.01 \cdot \left(\frac{K}{k_{+2}}\right) = \frac{4.6K}{k_{+2}} \qquad (20)$$

and

$$([D]t)_{0.5} = -\ln 0.5 \cdot \left(\frac{K}{k_{+2}}\right) = \frac{0.69K}{k_{+2}} \qquad (21)$$

The product $([D]t)_{0.5}$ is a unique value, but $[D]_{0.5}$ and $t_{0.5}$ are not. For each β-lactam compound concentration there is one $t_{0.5}$ value, and, similarly, there is one $[D]_{0.5}$ for each time of incubation. The same remark applies, of course, to the product $([D]t)_{0.99}$.

A direct corollary of eqn. (18) is that a 23-fold (or a 7.2-fold etc.) increase in the β-lactam compound concentration is sufficient to cause a change in the extent of enzyme acylation from 10% to 90% (or from 20% to 80% etc.). Hence, if a saturation curve (plot of extents of enzyme acylation versus β-lactam compound concentrations) fulfils the condition $[D]_{0.9}/[D]_{0.1} = 23$ (or $[D]_{0.8}/[D]_{0.2} = 7.2$ etc.), any extent of enzyme acylation and the corresponding β-lactam compound concentration can be used to compute the k_{+2}/K values on the basis of eqn. (18). In particular, the $[D]_{0.5}$ value can be converted into the k_{+2}/K value on the basis of eqn. (21). With the saturation curves 4, 3, 2 and 1 shown in Fig. 2, the $[D]_{0.9}/[D]_{0.1}$ ratio values are 23, 24, 47 and 80 (a limit $[D]_{0.9}/[D]_{0.1}$ ratio value of 81 is obtained for $t > 4000$ s). In parallel with this, the relevant $[D]_{0.5}$ values yield, on the basis of eqn. (21), k_{+2}/K values of 9600, 8600, 5900 and 1700 $M^{-1} \cdot s^{-1}$ (to be compared with a theoretical value of 10000 $M^{-1} \cdot s^{-1}$). Note that in the case of curve 4 a 100% (or close to 100%) level of enzyme acylation would be achieved, at the steady state of the reaction, at all those β-lactam compound concentrations that are able to acylate the enzyme by at least 10% in 50 s.

Practical procedures for determining k_a and k_{+2}/K. Determination of k_a, at a given [D] value, involves experiments through which acyl-enzyme formation can be monitored as a function of time. Various techniques have been used: trapping of radioactive E–D*; change in some physical properties of the protein, as, for example, fluorescence quenching; change in the absorption spectrum of the β-lactam compound; disappearance of enzyme activity (Frère *et al.*, 1975*a*; Fuad *et al.*, 1976).

When the procedure involves trapping of a radioactive acyl-enzyme, this latter is stabilized by treatment with a denaturing reagent and, after polyacrylamide-gel electrophoresis in the presence of SDS, the separated radioactive acyl-enzyme is detected by fluorography and its amount determined (Spratt, 1983).

It may happen that no direct procedure is available for determining k_a (in particular when the enzyme activity is difficult to monitor or when the β-lactam compound is not in a radioactive form). An indirect procedure can be used in which the enzyme, left free after a first incubation with various concentrations of the non-radioactive β-lactam compound, is fully acylated, in a second step, by a radioactive β-lactam compound that obeys eqn. (18) and for which the relevant k_{+3} and k_{+2}/K values are known. The $[D]_{0.5}$ value of the non-radioactive β-lactam compound then refers to the β-lactam compound concentration that decreases by 50% the amount of enzyme susceptible to be acylated by reaction with the radioactive β-lactam compound and is referred to as an exclusion dose value. In this indirect procedure, the non-radioactive β-lactam compound needs not to be eliminated from the reaction mixture provided that the radioactive β-lactam compound is used at a concentration $[D]_{0.99}$ such that $t_{0.99}$ in eqn. (20) is at least 20-fold smaller than the incubation time used for reaction with the non-radioactive β-lactam compound. For example, assume (i) that the non-radioactive β-lactam compound is a much more potent inactivator for a given enzyme ($k_{+2}/K = 100000$ $M^{-1} \cdot s^{-1}$) than the radioactive β-lactam compound ($k_{+2}/K = 1000$ $M^{-1} \cdot s^{-1}$), (ii) that k_{+3} is negligible with both β-lactam compounds, and (iii) that a 900 s incubation is used for reaction with the non-radioactive β-lactam compound. After this first incubation, the enzyme is saturated by 50% at a 7.7 nM non-radioactive β-lactam compound concentration (reaction 21). Trapping the enzyme left free can then be carried out in a second incubation with 100 μM radioactive β-lactam compound (i.e. a concentration at least equal to $4.6/1000$ $M^{-1} \cdot s^{-1} \times 45$ s). Indeed, in the absence of non-radioactive β-lactam compound, the free enzyme would be acylated by 99% in 45 s by the radioactive β-lactam compound. During these 45 s (and in the absence of radioactive β-lactam compound) the 7.7 nM non-radioactive β-lactam compound would acylate only 3% of the enzyme.

How should the inactivating potency of a β-lactam compound towards a given active-site-serine DD-peptidase be defined? The inactivating potency of a β-lactam compound is quantitatively expressed by the values of k_{+2}/K and k_{+3}, which govern the rate of enzyme acylation and the half-life of the acyl-enzyme respectively. As shown by eqn. (11), the higher k_{+2}/K and the smaller k_{+3}, the lower the β-lactam compound concentration for which $Kk_{+3}/k_{+2}[D]$ becomes negligible. When $Kk_{+3}/k_{+2}[D]$ is smaller than 0.01, virtually all of the enzyme is immobilized as acyl-enzyme at the steady state of the reaction ($[E–D^*]_{ss} = [E]_0$).

Reaction with a β-lactam compound may be characterized by a K_m value, often referred to as K_i, that, since $k_{+3} \ll k_{+2}$, simplifies to:

$$K_i = \frac{Kk_{+3}}{k_{+2}} \qquad (22)$$

K_i may also serve to express inactivating potency: the lower the K_i, the more potent the inactivator. However, if k_{+3} has a very small absolute value, K_i is necessarily very small even if k_{+2}/K is small, i.e. even if E–D* formation is a slow process. Hence at $[D] = K_i$ acylation of the enzyme may be so slow that it does not occur at all during the incubation time used and the enzyme remains fully active. At the limit when $k_{+3} = 0$ the concept of K_i has no practical value.

Penicillin-binding proteins: intrinsic resistance to and specificity index of β-lactam compounds. Assuming that all PBPs (see the Introduction) bind β-lactam antibiotics according to reaction (12), determination of the parameters involved has important implications. One of them relates to the degree of 'sensitivity' that a PBP

should exhibit in order to be a 'physiologically important target' for a given β-lactam compound in living bacteria. (i) Assume that the bacterium divides with an average generation time of 30 min, that the β-lactam compound concentration that is available at the level of the PBP target is 0.5×10^{-4} M and that $K = 10$ mM, $k_{+2} = 0.1\ s^{-1}$ (i.e. $k_{+2}/K = 10\ M^{-1} \cdot s^{-1}$) and $k_{+3} = 1 \times 10^{-4}\ s^{-1}$ (thus conferring on the acyl-PBP a half-life much longer than the generation time). Under these conditions the PBP is acylated by 83% at the steady state of the reaction (eqn. 11), it takes 128 min for the reaction to reach the steady state (eqn. 8) and in 30 min only 54% of the PBP is acylated. (This analysis is evidently an oversimplification. In a growing culture, the situation is much more complex owing to the continuous synthesis and turnover of the PBPs, the possible presence of a β-lactamase and the occurrence, at least in the Gram-negative bacteria, of an outer membrane, which functions as an efficient permeability barrier.) (ii) If k_{+3} does not have a very small absolute value, a large percentage of the PBP may escape acylation in the continuous presence of a β-lactam compound. Thus, for example, for $K = 10$ mM, $k_{+2} = 1\ s^{-1}$ (i.e. $k_{+2}/K = 100\ M^{-1} \cdot s^{-1}$), $k_{+3} = 1 \times 10^{-2}\ s^{-1}$ (half-life of the acyl-enzyme = 69 s) and [D] = 1×10^{-4} M, eqn. (11) shows that, although k_{+3}/k_{+2} is small (0.01), at all times about 50% of the PBP remains in a free form. The above examples are meant to illustrate cases of high intrinsic resistance to β-lactam compound action.

Another question arises. How much more sensitive than any other of the PBPs present should a PBP be in order to be a specific target of a given β-lactam compound? Assuming that saturation of the PBPs proceeds according to eqn. (18), then the ratios between the relevant k_{+2}/K values can be used as a 'specificity index' to express the ability of the β-lactam compound to discriminate between the various PBPs. In the case of a mixture of two PBPs, a ratio of 60 between the relevant k_{+2}/K values means that any condition causing 95% saturation of the most sensitive PBP causes 5% saturation of the less sensitive PBP. A 'specificity index' of 60 may thus be taken as the minimal value that permits selective inactivation of a PBP by a particular β-lactam compound (although, at present, the percentage of a PBP that should be acetylated in order to impair completely its physiological function remains unknown).

CONCLUSIONS

On the basis of reaction (1) for the interaction between the active-site-serine DD-peptidases and amide or ester carbonyl-donor substrates and of reaction (12) for the interaction between the same enzymes and β-lactam suicide substrates, the values of the parameters involved in the reactions can be determined by measuring the amounts of acyl-protein formed during the processes. With substrates, k_{+3} cannot exceed k_{+2} by more than one order of magnitude. Yet, for $k_{+3} \geqslant 10 \times k_{+2}$, $k_{cat.}$ approximates k_{+2} and K_m approximates the dissociation constant K of the Michaelis complex. Moreover, irrespective of the k_{+3} and k_{+2} values, the $k_{cat.}/K_m$ ratio is equivalent to the second-order rate constant k_{+2}/K of enzyme acylation.

In order to define correctly the 'affinity' of a PBP (DD-peptidase) for a β-lactam compound (see the Introduction), several principles must receive particular attention. The following points may seem self-evident. They have not always been observed in past studies and they are important to avoid misleading results. Valid data can be obtained only under the following conditions.

(1) The stability of the native PBP under study must be established.

(2) The PBPs preparation must be shown not to contain β-lactamase activity that may degrade the β-lactam compound. Alternatively, the β-lactamase must be inactivated.

(3) The β-lactam compound concentration must be much larger than the sum of the concentrations of all of the PBPs present. With very potent β-lactam compounds (very high k_{+2}/K values) it may occur that, given a too long incubation period (but this relatively 'long' time may be a matter of seconds), the low β-lactam concentration that is apparently necessary to acylate a PBP (DD-peptidase) by 50% is, in fact, equivalent to half the concentration of the PBP (DD-peptidase) present. (In this case, $[D]_{0.9}/[D]_{0.1} = 9$.) This 'titration effect' (Chase *et al.*, 1978; Frère *et al.*, 1979) has been used to develop a rapid and sensitive method for the detection of β-lactam compounds in various biological fluids (Frère *et al.*, 1980).

(4) The total amount of PBP under study (i.e. $[E]_0$) must be determined correctly (see also below).

(5) The value of k_{+3} must be determined.

(6) The k_a values at various β-lactam compound concentrations must be determined under conditions that assure that, at the given incubation time, the PBP is fully acylated at the largest β-lactam concentrations used. For the determination of k_a, eqn. (16) is general. Simplified eqn. (17) is valid if (i) the incubation time is at least 10-fold smaller than the half-life of the acyl-PBP, (ii) at those β-lactam compound concentrations that do not cause complete acylation of the PBP within the incubation time used, complete (or almost complete) PBP acylation would occur at the steady state of the reactions, and (iii) a 23-fold increase (or 7-fold increase) in the β-lactam compound concentration causes a change in the extents of PBP acylation from 10% to 90% (or from 20% to 80%).

(7) The k_a value obtained at a given β-lactam concentration must be independent of the incubation time. Several incubation intervals should be used. If only one incubation time is used, the investigator must be aware that computation of the k_a value rests upon a one-point kinetic (and thus has limited precision).

(8) From the k_a values obtained at several β-lactam concentrations, k_a versus [β-lactam compound] should be plotted. If linearity is observed, the slope of the line is equal to k_{+2}/K. If linearity is not observed, the reciprocal plot of $1/k_a$ versus 1/[β-lactam compound] or the plot of [β-lactam compound]/k_a versus [β-lactam compound] should give rise to a straight line (intercept = $1/k_{+2}$ or K/k_{+2}; slope = K/k_{+2} *or* $1/k_{+2}$).

Although exceptions exist, the reactions between PBPs (DD-peptidases) and β-lactam compounds usually generate a long-lived acyl-PBP (acyl-enzyme). At least in those cases where the problem has been examined (Ghuysen *et al.*, 1984; Frère & Joris, 1985), the K values range between 0.1 and 10 mM. It thus seems probable that many $[D]_{0.5}$ values published in the literature concerning the interactions between PBPs and β-lactam compounds were actually derived from saturation curves obtained at $[D] \ll K$ and $k_a \gg k_{+3}$. Hence, introducing the published $[D]_{0.5}$ values in eqn. (21) may give at least rough estimates

of the values of the second-order rate constant of PBP acylation.

Finally, determination of the k_{+2}/K and k_{+3} values is important in order to investigate the possible physiological role of a given PBP (DD-peptidase) and to define the selectivity of action of a given β-lactam compound at the level of the PBP (DD-peptidase) targets.

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