Interactions of cephalosporins with the *Streptomyces* R61 DD-transpeptidase/carboxypeptidase

Influence of the 3'-substituent

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The influence and fate of the 3'-substituent of a series of cephalosporins on their interaction with the DD-transpeptidase/carboxypeptidase of *Streptomyces* R61 were investigated. Good 3'-leaving groups are eliminated from the acyl-enzyme before deacylation, and hence cephalosporins with such substituents generate a common covalent intermediate. The rate of this elimination reaction in the two cases studied in detail was much lower than from the corresponding cephalosporoates in free solution. Cephalosporins without good 3'-leaving groups yield acyl-enzymes that appear to hydrolyse, leading to enzyme re-activation, faster than those from cephalosporins with leaving groups.

INTRODUCTION

It has been recently shown [1, 2] that even good leaving groups, X, at the 3'-position (Scheme 1) of the important cephalosporin antibiotics (I) need not depart in a concerted fashion during nucleophilic β -lactam cleavage. The alternative stepwise elimination pathway has interesting and important consequences with respect to cephalosporin turnover (Scheme 1) by β -lactamases [3, 4] since, in certain cases at least, the acyl-enzymes (III) generated through the elimination reaction at the enzyme active site appear to be more inert to hydrolysis than their parents (II). Diversion of (II) to (III) can thus retard destruction of the cephalosporin by the β -lactamase and, potentially at least improve the drug's antibiotic effectiveness. Boyd [5] has proposed that biological data do indicate that the presence of a leaving group at the 3'-position of a cephalosporin does produce a more

effective antibiotic, although the mechanism of this effect has not yet been directly demonstrated.

Antibiotic effectiveness must of course also be strongly influenced by the nature of the interaction of the cephalosporin with the site of antibiotic activity, the bacterial cell-wall D-alanyl-D-alanine (abbreviated DD)-transpeptidases. The most detailed chemical models of this process, developed by Ghuysen, Frère and co-workers [6, 7], have involved the soluble enzymes excreted by certain *Streptomyces* strains, and in particular that from strain R61. The X-ray-crystallographic structure of the R61 enzyme is under active investigation by Knox, Kelly and co-workers. Although preliminary difference maps, with cephalosporin C bound, suggested [8] that the 3'-acetoxy group might still be present in the stable enzyme-substrate complex, closer investigation [9] made this seem less likely. Although the kinetics of interaction of this enzyme with a wide variety of β -lactam antibiotics

Scheme 1. Pathways of cephalosporin hydrolysis, catalysed by β -lactamases and the R61 DD-peptidase

R = thienyl; compound (Ia), X = OAc; compound (Ib), X = OH; compound (Ic), X = H; compound (Id), X = 2-(4'-dimethylaminophenylazo)pyridinium; compound (Ie), X = 3,4-dimethylpyridinium; compound (If), X = SPh; compound (Ig), X = 1-N-methyltetrazolyl-thio; compound (Ih), X = dansylamido (i.e. 5-dimethylaminonaphthalene-1-sulphonamido).

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have been carefully determined [7, 10, 11], there does not seem to have been any systematic investigation of the effect of the 3'-substituent alone. In this paper we present data on the fate and influence of a variety of 3'-substituents, commonly found in clinically important antibiotics, on the interaction of cephalosporins with the R61 enzyme; in the series employed, the 7-substituent is uniformly thienylacetamido.

EXPERIMENTAL

The soluble DD-transpeptidase/carboxypeptidase of *Streptomyces* R61 was generously provided by Dr. J.-M. Ghuysen and Dr. J.-M. Frère. The cephalosporins cephalothin (Ia), deacetylcephalothin (Ib), deacetoxycephalothin (Ic) and Keflet (Ig) were gifts from Eli Lilly and Co., and PADAC (Id) was a gift from Hoechst-Roussel Pharmaceuticals. The preparations of 3,4-dimethylcephaloridine (Ie) and thiophenoxycephalothin (If) are described elsewhere [2], as is that of dansylcephalothin (Ih) [12].

All experiments were done in 20 mM-potassium phosphate buffer, pH 7.0, at 37 °C. The activity and concentration of the R61 enzyme were assayed spectrophotometrically at 254 nm with hippuryl-DL-phenyl-lactate (Sigma Chemical Co.) as substrate (C. P. Govardhan & R. F. Pratt, unpublished work), and with the assumption that the molar absorption coefficient of the enzyme was $3.90 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13].

Pseudo-first-order rates of acylation (and inactivation) of the enzyme were measured by monitoring loss of the cephem chromophore. Thus to 200 μl of an R61 enzyme solution (final enzyme concentration 5.0–9.5 μM) was added 5.0 μl of a cephalosporin solution, yielding final cephalosporin concentrations in the range 40–200 μM , and the subsequent reaction was monitored spectrophotometrically at 260 nm. These measurements yielded a burst amplitude and a pseudo-first-order rate constant. Under these conditions a plot of the observed pseudo-first-order rate constants against cephalosporin concentration was linear in each case, yielding k_2/K_1 [see eqn. (1) below] as slope.

Inert acyl-enzymes could be isolated by exclusion chromatography at 4 °C. Solutions of cephalosporins (5 μl) were added to 200 μl portions of an R61 enzyme solution and the mixtures were incubated at 37 °C until the reactions were complete. Final enzyme concentrations were 20–45 μM and cephalosporin concentrations 280, 600 and 300 μM for (Id), (Ig) and (Ih) respectively. After inactivation, the solutions were loaded on to a Sephadex G25–40 column (0.5 cm $\times$ 13.5 cm) at 4 °C and eluted with 20 mM-phosphate buffer at the same temperature. Fractions (0.4 ml) were collected and assayed for protein by their absorbance at 280 nm. They were also assayed spectrophotometrically for cephem nucleus after treatment with alkali, as previously described [3]. The amount of the 3'-substituent still bound to the enzyme in the protein fractions was determined in each case by difference absorption spectra, and also by dansyl fluorescence (λ_{exc} , 330 nm) in the case of (Ih) and by assay with Ellman's reagent [14] (see below) in the case of (Ig).

In order to measure rates of re-activation, 5 μl of a cephalosporin solution, to a final concentration of 200–600 μM , depending on the cephalosporin, was added to 200 μl of an R61 enzyme solution (final enzyme

concentration 6–9 μM), and the reaction was allowed to proceed until the enzyme was completely inactive. Excess cephalosporin was then destroyed by the addition of 1.0 μl of a TEM-2 β -lactamase solution (final concentration 0.1 μM), and the R61 enzyme activity of 15 μl samples was measured as a function of time. Typically the re-activation process was monitored until it was somewhere between 25 and 50% complete. Rate constants were calculated assuming a first-order reaction with the activity at infinite time that of the original enzyme. The rate constants reported were reproducible within 10%.

RESULTS AND DISCUSSION

The acyl-enzyme formed on incubation of the R61 DD-transpeptidase/carboxypeptidase and PADAC (Id) was found to contain 1.0 mol of cephem/mol of enzyme and none of the chromophoric 3'-substituent. The inactive covalent ES complex in this case had clearly lost the 3'-leaving group, and thus had structure (III).

The kinetics of interaction of cephalosporins (Ia)–(Ih) with the R61 enzyme are shown in Table 1. These results are interpreted in terms of the established [6, 7] scheme of turnover of β -lactam antibiotics by this enzyme (eqn. 1):

where ES is the Michaelis complex and E-S the acyl-enzyme. They show firstly that the 3'-substituent influences enzyme acylation (k_2/K_1) in a way not predictable solely on the basis of electronic effects of the 3'-substituent [10, 15]. In particular, the pyridinium cephalosporins (Id) and (Ie) acylate the enzyme much more slowly (k_2/K_1) than would be expected on the basis of the electron-withdrawing ability of their substituents with respect to the others. This might indicate an unfavourable effect of the positively charged pyridinium substituent on the structure and thus activity of the active site; this could, for example, stem from an electrostatic interaction with the (presumably) positively charged lysine side chain that Kelly *et al.* [9] find adjacent to the 3-position in the complex of cephalosporin C with the R61 enzyme. It is noteworthy that 3'-pyridinium cephalosporins also appear to have a low affinity for class C β -lactamases [16].

The effect of the 3'-substituent on deacylation is seen in the k_3 values of Table 1, where the values for primary comparison are those for PADAC, where the acyl-enzyme has structure (III), and for (Ic), which does not have a 3'-leaving group, and whose complex with the R61 enzyme presumably has the structure (II). The k_3 values for (Ia) and (Ig) are the same as that for (Id), suggesting that the same acyl-enzyme is present, and hence that the good 3'-leaving groups present on these compounds also depart rapidly after formation of the first acyl-enzyme (II); the same result is obtained on interaction of these cephalosporins with the *Staphylococcus aureus* PC1 β -lactamase [3].

The significantly different deacylation rates found with compounds (Ib), (Ic), (If) and (Ih) suggest that these compounds form complexes with the enzyme that are different from those discussed above. Their likely structure is (II), where the potential leaving group has not departed. Some other evidence for this is as follows.

Table 1. Kinetics of interaction of cephalosporins with the R61 enzyme

All experiments were performed in 20 mM-potassium phosphate buffer, pH 7.0, at 37 °C, unless otherwise noted. k_2/K_1 and k_3 are constants of eqn. (1). k_4 and k_5 are constants of Scheme 1.

Cephalo- sporin	3'-Substituent	k_{25}/K_1 ($M^{-1} \cdot s^{-1}$)	$10^6 \times k_3$ (s^{-1})	k_4 (s^{-1})	k_5 (s^{-1})
(Ia)	OAc	350	3.2	$> 0.07\parallel$	$> 50\parallel$
(Ib)	OH	390	5.7	$< 5.7 \times 10^{-6}$	0.016**
(Ic)	H	83	5.3	0	0
(Id)	py ⁺ *	11	3.1	$> 0.003\parallel$	11 $\parallel$
(Ie)	py ⁺ †	26	5.54	$> 0.001\parallel$	0.055**
(If)	SPh	500	11.2 (3.3††)	5.8×10^{-4}	0.24
(Ig)	SAr‡	260	3.05	$> 0.03\parallel$	> 50
(Ih)	NHDNS§	685	16.4	0	0

* 2-(4'-Dimethylaminophenylazo)pyridinium.

† 3,4-Dimethylpyridinium.

‡ 1-N-Methyltetrazolyl-5-thio.

§ Dansylamido.

$\parallel$ Lower limit from fastest acylation rate observed.

$\parallel$ 20 °C; reference [3].

** 30 °C; reference [2].

†† In the presence of Ellman's reagent (see the text).

Firstly, in the case of (Ib), the absorbance change at 260 nm ($\Delta\epsilon = 4100 M^{-1} \cdot cm^{-1}$) corresponds to that expected in formation of (II) rather than (III) [2]. Secondly, in the case of (If), the difference spectrum of the isolated acyl-enzyme compared with the native enzyme showed a shoulder at 260 nm ($\epsilon = 2500 M^{-1} \cdot cm^{-1}$), typical of structure (II) [2], and not expected with structure (III). Also, addition of Ellman's reagent (300 μM) led to first-order ($5.8 \times 10^{-4} s^{-1}$) formation of 1 equivalent (with respect to the enzyme) of the 2-nitro-5-mercaptobenzoate dianion, suggesting dissociation of (II) to (III) plus thiophenol, which was then trapped by the thiol reagent. This interpretation is supported by the re-activation rates of the complex with (If), shown in Table 1. In the absence of Ellman's reagent the rate is significantly higher than those of (Ia), (Id) and (Ig), but after incubation of the enzyme with this reagent (300 μM) and (If) together the re-activation rate fell to that of these compounds, indicating that the leaving group had departed and (III) had been again generated. Finally, in the case of (Ih), the isolated acyl-enzyme retained dansyl fluorescence of appropriate intensity.

Thus the rates of hydrolysis of (III) are different from and, significantly, in the cases studied here, rather less than those of (II). This is again the same result as that found in several instances with β -lactamases [3, 4], although the difference between the hydrolysis rates of (II) and (III) seems rather less dramatic here.

Note that the above results imply that (IIe) and (II) are in equilibrium with (III) under the conditions of the re-activation reaction. Model studies [2] indicate that this is not unexpected. Because of this fact, however, the values of k_3 reported for these compounds in Table 1 may be less than the true values. Experiments at higher nucleophile concentrations (W. S. Faraci & R. F. Pratt, unpublished work) suggested that the saturation values of k_3 were $5.6 \times 10^{-6} s^{-1}$ and $3.6 \times 10^{-5} s^{-1}$ for (IIe) and (II) respectively. Compound (IIb) is probably not at equilibrium with respect to (III), since, if this were true,

compounds (Ia), (Id) and (Ig) should also yield (IIb) after elimination and water addition; that they do not do so is clear from the re-activation rates.

The effect of the enzyme on the dissociation rates of X from the acyl-enzymes is also shown in Table 1. In the cases where a definite conclusion is possible, (IIb) and (II) [unfortunately, the rates for (II) and (IIe) could not be determined because of the slow acylation of the enzyme by (Id) and (Ie)], the elimination rate is markedly decreased, by 400-fold in the latter case, and at least 5000-fold in the former. These decreases could reflect improper orientation of the bound 3'-substituent for ready elimination [17], and, also, in the case of (IIb) perhaps, a much lower basicity, than in (IV), of the hydroxy-group oxygen atom, whose protonation is probably necessary for elimination [2]; the latter effect may also be brought about by an adjacent lysine cation.

In summary, these results indicate that ready elimination of good leaving groups from the 3'-position in acyl-enzymes generated on interaction of the R61 DD-transpeptidase/carboxypeptidase with cephalosporins does occur. There is the further suggestion, from the observation with poorer leaving groups, that the enzyme may, in general, strongly influence the rates of the elimination reaction. Elimination of the leaving group does appear, as with β -lactamases [3, 4], although to a lesser extent in the cases observed to date, to generate a more hydrolytically inert acyl-enzyme, and thus, potentially at least, to lead to a more effective antibiotic. Although the relative antibiotic efficiency of β -lactams towards an R61-like enzyme would probably be dictated by acylation rates (k_2/K_1) rather than deacylation (k_3) [6, 7], this might not be true of all DD-transpeptidases.

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