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NOTES

CENTA as a Chromogenic Substrate for Studying β-Lactamases

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CENTA, a chromogenic cephalosporin, is readily hydrolyzed by β-lactamases of all classes except for the *Aeromonas hydrophila* metalloenzyme. Although it cannot practically be used for the detection of β-lactamase-producing strains on agar plates, it should be quite useful for kinetic studies and the detection of the enzymes in crude extracts and chromatographic fractions.

Nitrocefin and, to a lesser extent, PADAC have been used as chromogenic substrates of β-lactamases. Such substrates, whose hydrolysis can be directly monitored in the visible wavelength range, are of particular interest for the kinetic characterization of β-lactamases. Nitrocefin has, for instance, been widely used as a reporter substrate in the study of the inactivation of β-lactamases or of their interactions with poor substrates (3). It also allows the rapid identification of active fractions during β-lactamase purification. However, the price of nitrocefin has recently been increased significantly, and PADAC is no longer commercially available. Synthesis of compounds is also rather tedious. It is thus surprising that a third chromogenic cephalosporin, CENTA (Fig. 1), which can be prepared from the commercially available drug cephalothin, has not received more attention, although it was shown to be sensitive to many β-lactamases (11). In the study described in this report, we determined the kinetic parameters characterizing the interactions between CENTA and a representative set of β-lactamases and some penicillin-binding proteins (PBPs). Although CENTA cannot be used for the direct detection of β-lactamase-producing colonies on agar plates, it still represents an interesting alternative to nitrocefin for the kinetic characterization of β-lactamases.

CENTA was prepared as follows: 3-carboxyl-4-nitrothiophenol (TNB) was obtained by dissolving 5.05 mmol (2 g) of 5,5′-dithio-bis-(2-nitrobenzoic acid) in 100 ml of an aqueous solution of 0.5 M Tris base, and the pH was adjusted to 8.0 by addition of 6 M HCl. Dithiothreitol (7.1 mmol, 1.1 g) was added, and the solution turned orange-red. The mixture was stirred for 10 min at 22°C and extracted six times with 25 ml of ethyl acetate before being acidified to pH 1.5 by addition of 6 M HCl. The residual ethyl acetate was eliminated by bubbling nitrogen through the solution, which was thereafter left overnight at 4°C. The precipitate (TNB) was collected by filtration, washed, and dried. The sodium salt of cephalothin (1 g, 2.4 mmol) and 1 equivalent of TNB (478 mg) were dissolved in 19 ml of H_2O, and the pH was adjusted to 7.0 with 1 M NaOH. The solution was stirred for 6 h at 65°C. The cooled solution was extracted with 10 ml of ethyl acetate, acidified to pH 2.0 with 1 M HCl, and extracted three times with 15 ml of ethyl acetate. The organic phase was washed three times with 15 ml of water, dried over $MgSO_4$, and evaporated to dryness in vacuo. The sodium salt of CENTA was obtained by dissolving the dry residue in 25 ml of water containing 1 equivalent of $NaHCO_3$, and the solution was freeze-dried, yielding 1.36 g of a light brown solid. Filtration through a Sephadex G-10 column did not modify the yield or improve the purity of the compound, as demonstrated by infrared spectroscopy (KBr) or nuclear magnetic resonance imaging at 200 MHz. The final yield was 1.31 g (94%, with respect to cephalothin).

CENTA was tested as a substrate for class A (TEM-1 [17], NMCA [19], SHV-1 [1], TOHO-1 [10], *Mycobacterium tuberculosis* [22], *Staphylococcus aureus* [21]), class B (*Bacillus cereus* [2], CphA [9], IMP-1 [12], CfiA [16], BlaB [18], VIM-1 [5]), class C (*Enterobacter cloacae* 908R [7], *Pseudomonas aeruginosa, Citrobacter freundii*, and ACT-1), and class D (OXA-10 [1], OXA-2 [13]) β-lactamases and as an inactivator of the soluble form of the *Escherichia coli* PBP 5 (20), *Streptomyces* sp. R61 DD-peptidase (8), *Actinomadura* sp. R39 DD-peptidase (8), and *Streptomyces* sp. K15 DD-transpeptidase (14). All kinetic experiments with β-lactamases and PBP 5 were performed at 30°C in 50 mM sodium phosphate (pH 7.0) with 100 μM $ZnSO_4$ added for the *B. cereus*, IMP-1, and CfiA class B metallo-β-lactamases. The hydrolysis of CENTA was monitored by continuously recording the absorbance variation at 346 nm ($\Delta\varepsilon = -2{,}500\ M^{-1}\ cm^{-1}$) or 405 nm ($\Delta\varepsilon = +6{,}400$

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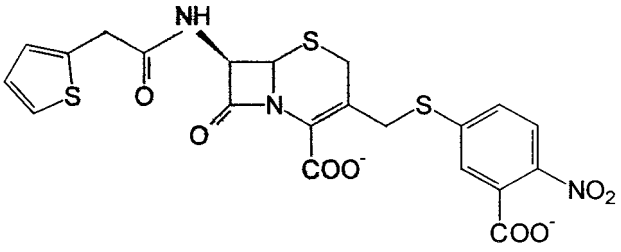

FIG. 1. Structure of CENTA.

M^{-1} cm^{-1}). The k_{cat} and K_m values were derived from initial rate measurements with the help of the Hanes linearization of the Henri-Michaelis equation and direct fitting on the hyperbolic equation by nonlinear regression or from complete time courses (3). The lowest K_m values obtained with SHV-1, CfiA, OXA-10, and OXA-2 were verified by using CENTA as a competitive inhibitor versus 100 μM nitrocefin for the first three enzymes and versus 300 μM cefaclor for the fourth one. The other experimental conditions are detailed in Table 1.

Inactivation of the R61, R39, and K15 DD-peptidases was monitored by incubating the enzymes with various concentrations of CENTA and measuring the residual activity of an aliquot after increasing periods of time. Activity was determined by measuring the production of D-alanine from N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine by the D-amino acid oxidase method (6). In the case of the K15 enzyme, the assay mixture also contained 10 mM glycylglycine. The buffers were as follows: for R39, 50 mM Tris (pH 8.0) plus 1 mM $MgCl_2$; for R61 and K15, 50 mM Tris (pH 8.0).

Table 1 summarizes the kinetic constants obtained with CENTA and the various β-lactamases and compares them with those obtained with nitrocefin. CENTA was a relatively good substrate of all the enzymes with the sole exception of the CphA enzyme, which is very specific for carbapenems and similarly exhibits very poor activity against nitrocefin. The kinetic parameters of OXA-10 and SHV-1 with nitrocefin were determined in the present work. When compared to nitrocefin, the initial rates recorded at the same 100 μM concentration are of the same order of magnitude with the exceptions of those for the OXA-10 and SHV enzymes. One should be reminded, however, that the Δε value of CENTA (+6,400 M^{-1} cm^{-1}) is significantly lower than that of nitrocefin (+17,500 M^{-1} cm^{-1}). Nonetheless, CENTA can easily be used for monitoring the presence of all the enzymes (excepted CphA) in chromatographic fractions and as a reporter substrate for detailed kinetic studies even with enzymes (*M. tuberculosis* and OXA-10) which exhibit rather low levels of activity against this compound. The maximum change of absorbance of the leaving group is different from that for most β-lactam antibiotics. Interestingly, and in contrast to nitrocefin, hydrolysis of CENTA

TABLE 1. Kinetic parameters for interaction between active-site serine and zinc β-lactamases and CENTA of nitrocefin[a]

Enzyme group and enzyme	CENTA						Nitrocefin				$(v_0)_N/(v_0)_C$[c]
	Methods	[CENTA] (mM)	[Enz] (nM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	Reference[b]	
Class A β-lactamases											
TEM-1	v_0	0.025–0.3	11.5	110	70	1.6	930	52	18	17	9.4
NMCA	v_0	0.25–1.5	7	340	100	3.4	88	104	0.84	19	0.25
SHV-1	CTC + RS	0.25–1	1.4–170	11	27	0.4	88	104	0.84	1	32
TOHO 1	v_0	0.25–1.5	1.8	2,000	180	11	520	60	9	10	0.45
M. tuberculosis	v_0	0.25–1.5	310	5	120	0.04	31	80	0.4	22	7.5
S. aureus	v_0	0.01–0.1	77	2	10	0.2	16	1.5	10	4	8.7
Class C β-lactamases											
908R	CTC	0.03	10	110	10	11	780	23	34	7	6.3
P. aeruginosa	CTC	100–200	25–50	390	63	6.3	740	27	27	7	ND[d]
C. freundii	CTC	100–200	50–90	75	25	3	330	12	28	7	ND
ACT-1	CTC	100–200	1–2	690	80	8.6	ND	ND	ND	ND	ND
Class D β-lactamases											
OXA-10	CTC + RS	0.1–1	14–1,000	0.4	9	0.04	3,000	450	6.6	13	1,500
OXA-2	CTC + RS	0.025–0.5	4.8	95	13	7.3	Burst complex kinetic	Burst complex kinetic	Burst complex kinetic	13	ND
Class B β-lactamases											
BcII	v_0	0.25–1.5	14	50	330	0.15	45	70	0.64	16	2.3
CphA	v_0	0.1	6,000	NH	NH	NH	0.31	100	0.0003	9	ND
IMP-1	v_0	0.25–1.5	1	400	200	2	63	27	2.3	12	0.37
CfiA	CTC + RS	0.025–0.5	2.5–10	40	7	5.7	200	16	12.5	23	4.7
BlaB	v_0	0.05–0.4	14	4.5	31	0.15	17	66	0.26	3	3
VIM-1	v_0	0.05–2.2	1.8	430	600	0.7	95	17	5.6	5	ND

[a] Complete time courses (CTC) were recorded over 3- to 15 min periods; initial rate (v_0) values were recorded over 1 min. With CphA, no hydrolysis (NH) of 100 μM CENTA could be detected after 30 min (initial rate, <5 μM h^{-1}). The low K_m values were measured as K_is with the help of a reporter substrate (RS) (usually 100 μM nitrocefin, with the exception of the OXA-2 enzyme, with which 300 μM cefaclor was used). Standard deviation values did not exceed 10%.

[b] Kinetic data for nitrocefin were found in the cited references.

[c] The ratio between the expected initial rates for the hydrolysis of 100 μM nitrocefin [$(v_0)_N$] and 100 μM CENTA [$(v_0)_c$] by the same amount of enzyme.

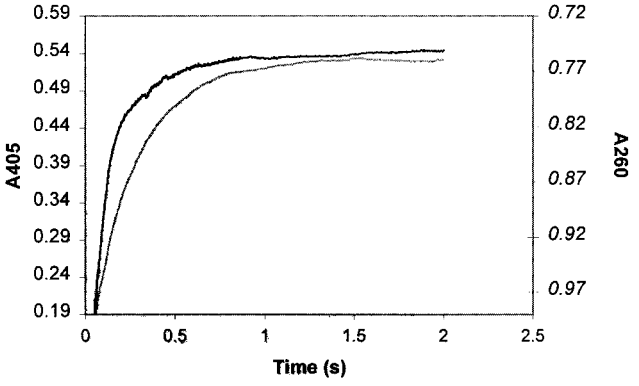

FIG. 2. Complete hydrolysis of 60 μM CENTA by 20 μM *E. cloacae* 908R β-lactamase. The light line shows the increase in the A_{405}, and the heavy line shows the decrease in the A_{260}. The ordinate on the right (A_{260}) has been inverted to facilitate the comparison. The optical pathway of the cell was 1 cm.

by the OXA-2 class D enzyme does not exhibit the burst phenomenon which precludes the use of the latter substrate in competition and reporter substrate experiments.

Although it was a poor substrate and inactivator of PBP 5 (k_{cat}/K_m = 22 M^{-1} s^{-1}), the k_{cat} value was unexpectedly high for a PBP (5 × 10^{-3} s^{-1}), reflecting a relatively rapid deacylation step (>5 × 10^{-3} s^{-1}). The K_m value was, accordingly, rather high (220 μM). With the *Streptomyces* sp. R61 and *Actinomadura* sp. R39 enzymes, the second-order inactivation rate constants were of the same order of magnitude as those observed with cephalothin (2,000 ± 200 and 90,000 ± 10,000 M^{-1} s^{-1}, respectively). Deacylation was very slow in both cases (<10^{-4} s^{-1}). Finally, the K15 enzyme was not sensitive to CENTA ($k_2/K' < 0.1$ M^{-1} s^{-1}), a result which reflects the low sensitivity of this enzyme to cephalothin (k_2/K' = 2 M^{-1} s^{-1} [14]).

Since the appearance of the chromophore is related to the expulsion of the C-3′ leaving group which is not concomitant with the opening of the β-lactam ring (4), it was important to verify that this expulsion was sufficiently rapid so that no artifact would be introduced in the measurement of initial rates or complete time courses. To do so, CENTA (30 to 60 μM) was hydrolyzed at 30°C with increasing concentrations of the *E. cloacae* 908R class C β-lactamase (0.03 to 20 μM). The variation in the absorbance was monitored at 260 nm (hydrolysis of the endocyclic amide bond) or 405 nm (appearance of the expulsed chromophore) for 1 to 80 s on a stopped-flow spectrophotometer (Biologic SFM-3; Grenoble, France). Figure 2 shows that a clear lag becomes detectable in the curve for 405 nm when the reaction is completed within about 1 s. From these data, it can be estimated that the first-order rate constant characterizing the expulsion step is ≥5 s^{-1} and that, as a consequence, the rate of this reaction is unlikely to influence the values of the rate constants derived from complete time courses recorded over at least 1 min or from initial rate measurements performed over at least 30 s. Accordingly, the increase in the A_{405} value was linear under conditions in which about 5% of a 200 μM CENTA solution was hydrolyzed in 1 min, and within the limits of experimental errors, the initial rates were the same when derived from measurements obtained when the absorbance was monitored at 405 or 260 nm.

The chemical properties of CENTA are also favorable. In contrast to nitrocefin, whose stock solution must be prepared in dimethyl sulfoxide or dimethylformamide, CENTA is highly soluble in aqueous buffers. At pH 7 and in 200 mM sodium phosphate buffer, it was soluble up to a final concentration of 60 mg/ml. The stability of a 100 μM solution was analyzed at a pH range of 4 to 12 at 25°C. The following buffers were used: for pH 4 and 5, 50 mM sodium acetate-acetic acid; for pH 6, 10 mM sodium cacodylate-HCl; for pH 7, 50 mM sodium phosphate; for pH 8, 10 mM Tris-HCl and 10 mM HEPES-NaOH; for pH 9 and 10, 50 mM CAPSO-NaOH; for pH 11, 50 mM Na_2HPO_4-NaOH; for pH 12, 50 mM KCl-NaOH. The absorbance at 405 nm was determined after increasing periods of time. When the incubation was performed below pH 7, the reading was made after adjustment of the pH of an aliquot to pH 7. Up to pH 9, no significant spontaneous hydrolysis of CENTA could be detected after a 60-min incubation. At higher pH values, the compound was less stable and the hydrolysis rates constants were 0.7 × 10^{-5} s^{-1} at pH 11 and 1 × 10^{-3} s^{-1} at pH 12. At the latter pH, the rate of hydrolysis of nitrocefin was 2.6 × 10^{-3} s^{-1}. Incubation of 100 μM CENTA in rabbit serum diluted fourfold at pH 7 and 30°C did not result in significant hydrolysis after 1 h, whereas the half-life of nitrocefin was 13 min under the same conditions (15). Similarly, at 30°C, substantial aminolysis of CENTA was observed in the presence of 300 mM Tris-HCl (pH 8.0; half-life, 19 min [the half-life for nitrocefin is 38 min]), but no detectable degradation occurred in the same buffer at a 10 mM concentration, in which nitrocefin exhibited a half-life of 380 min.

These experiments demonstrate that CENTA is a readily obtained chromogenic substrate which can conveniently be used in kinetic studies of β-lactamases and for the detection of these enzymes in bacterial crude extracts or in chromatographic fractions during enzyme purification. It can also be easily used in high-throughput screening tests for the selection of new β-lactamase inactivators. Unfortunately, the absorption spectrum of the leaving group is such that the contrast is not sufficient for the direct detection of β-lactamase-producing colonies on agar plates or on paper strip tests or for the localization of β-lactamases after gel isoelectric focusing.

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