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Anomalous dissociation constants for penicillinase and competing substrates

The binding of penicillins by penicillinase (penicillin amidohydrolase, EC 3.5.2.6) is accompanied by a reversible change in the conformation of the active site of the enzyme (conformative response)[1–4]. A quantitative study of the conformative response in four species of penicillinase has indicated that termination of the conformative response (relaxation) follows the dissociation of the enzyme–substrate complex. The time-scale of the relaxation step, however, may be sufficiently slow to affect the kinetics of the enzyme reaction[5]. In this communication we record an anomalous relationship between the apparent dissociation constants obtained from single substrate and mixed substrate determinations. The anomaly is attributed to the effect of the conformative response and can be eliminated by the use of specific antibodies which stabilize the conformation of the enzyme.

The penicillinase preparations were derived from three strains of *Bacillus cereus* (569, 569/H and 5/B) and purified as previously described[6]. Enzyme assays were carried out by the manometric[7] and by the alkalimetric[8] methods. The penicillins used were benzylpenicillin and two of its analogs, methicillin (6-(2,6-dimethoxybenzamido)-penicillanic acid) and oxacillin (6-(5-methyl-3-phenyl-4-isoxazolyl)-aminopenicillanic acid). The relative rates of hydrolysis of these compounds by the three penicillinases and the respective K_m values are listed in Table I.

It has been shown that the analogs which are hydrolyzed at a relatively slow rate (Table I), act as competitive inhibitors of the hydrolysis of benzylpenicillin[2,3] and induce a conformative response which differs markedly from that induced by the penicillin[6,8]. The effect of an analog-induced conformative response on the affinity of the enzyme for benzylpenicillin, and the reciprocal effect of benzylpenicillin, were investigated as follows.

Apparent dissociation constants derived from mixed substrate assays, involving benzylpenicillin and one of its analogs, were compared with the K_m values obtained

TABLE I

RELATIVE RATES OF HYDROLYSIS AND K_m VALUES FOR *B. cereus* PENICILLINASES AND THEIR SUBSTRATES

Rate of hydrolysis of benzylpenicillin by each penicillinase preparation = 100.

Enzyme	*Substrate*	*Relative* v_{max}	K_m *(mM)*
569/H	Benzylpenicillin	100	0.060
	Methicillin	3	0.46
	Oxacillin	5	0.57
569	Benzylpenicillin	100	0.058
	Methicillin	3	0.41
	Oxacillin	5	0.55
5/B	Benzylpenicillin	100	0.043
	Methicillin	3	0.23
	Oxacillin	4	0.33

from single-substrate determinations. Significant discrepancies between the two sets of constants were taken to indicate that the conformative response induced by one substrate molecule modifies the affinity of the enzyme for the competing substrate molecule.

The single-substrate constants for benzylpenicillin (K_m^S) and for its analogs (K_m^A) were determined directly by the graphical procedure of LINEWEAVER AND BURK[9]. The mixed-substrate constants were determined by two independent procedures.

First the analogs were considered as competitive inhibitors of hydrolysis of benzylpenicillin, and inhibitor constants (K_i) were determined from the inhibited reaction rates, corrected for the hydrolysis of the inhibitor[10].

The reciprocal values of the rates were plotted against inhibitor concentrations to give the K_i values directly, as suggested by DIXON[11]. It has been pointed out[12] that in this plot each slope cuts the base-line at a value [A] equal to $-K_i([S]/K_m + 1)$. It was thus possible to derive K_m^S values from the mixed-substrate data. The K_i and K_m^S values obtained by this procedure are listed in Table II. It will be noted that the K_m^S values are much higher than the Michaelis constants (benzylpenicillin) listed in Table I, and that they change considerably when methicillin is replaced by oxacillin as the inhibitor.

In an alternative procedure each analog was considered as a substrate competing with benzylpenicillin for the enzyme. It has been shown[13] that for an equimolar mixture of two such competing substrates, the ratio of the respective Michaelis constants can be obtained from the equation $K_m^S/K_m^A = (v_s - v_m)/(v_m - v_a)$ where v_s, v_a and v_m represent the maximum initial rates of hydrolysis of S, A and an equimolar mixture of S and A, respectively.

The ratios obtained by this procedure are compared in Table II with the ratios of the corresponding Michaelis constants derived from single-substrate determinations. The discrepancy between the two sets of values is obvious. A consequent and further discrepancy will be noted when K_m values derived from single-substrate determinations (Table I) are substituted in the observed ratios to obtain K_m values for the competing substrates.

In contrast, substitution of mixed-substrate constants in the ratios obtained

TABLE II

APPARENT DISSOCIATION CONSTANTS ($\times 10^{-3}$ M) DERIVED FROM MIXED SUBSTRATE DETERMINATIONS

Figures in parentheses in the first column represent the expected ratios, based on single-substrate determinations (see Table I); figures in parentheses in the third column represent constants derived from single-substrate determinations.

Enzyme	*Substrates*	K_m^S/K_m^A	K_m^S	K_m^A	K_i
569/H	Benzylpenicillin + methicillin	0.24 (0.13)	0.43	1.8 (0.46)	1.2
	Benzylpenicillin + oxacillin	0.33 (0.11)	2.0	6.0 (0.57)	5.0
569	Benzylpenicillin + methicillin	0.23 (0.12)	0.44	1.9 (0.41)	2.1
	Benzylpenicillin + oxacillin	0.31 (0.09)	1.4	4.5 (0.55)	3.5
5/B	Benzylpenicillin + methicillin	1.00 (0.19)	1.6	1.6 (0.23)	1.8
	Benzylpenicillin + oxacillin	1.25 (0.13)	2.4	1.9 (0.33)	2.6

from mixed-substrate determinations, yields consistent results. Thus, the K_m^S values obtained from Dixon plots (Table II) have been substituted in the ratio of $K_m^S : K_m^A$ obtained from equimolar mixtures (Table II) to give the K_m^A values listed in col. 3 of Table II. As expected, the K_m^A values thus obtained are fairly close to the corresponding K_i values (Table II). Here again the values are much higher than the corresponding Michaelis constants obtained directly from single-substrate determinations (Table I).

Studies now in progress indicate that the discrepancy between the two sets of constants is eliminated when the conformation of the enzyme is stabilized. A specific tool for stabilizing the conformation has been suggested by the observation[14] that immune serum prepared against 569/H penicillinase contains antibodies which effectively protect the enzyme against inactivation by heat, urea or proteolysis. Significantly, the antibodies suppress the conformative response of the enzyme. Moreover, the Michaelis constants for antibody-bound enzyme preparations are not affected by the presence of competing substrates, and the K_i values are consistent with the relevant Michaelis constants of the analogs. A detailed account of the effect of specific antibodies on the conformative response and kinetics of penicillinase will be published elsewhere[14].

In summary, we determined the Michaelis and inhibitor constants for three penicillinases and three penicillins. The values obtained in the presence of a competing substrate were considerably higher than those derived from single-substrate determinations. We suggested that the discrepancy reflects the previously established difference in the effect of each of the competing substrates on the conformation of the active site of the enzyme (conformative response). Thus, the conformative response induced by one substrate molecule appears to reduce the affinity of the enzyme for the competing substrate molecule. This suggestion is further supported by the observation that specific antibodies which suppress the difference in the conformative response eliminate the effect of the competing substrate on the kinetics of penicillinase.

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