STUDIES ON THE MODE OF ACTION OF AMICLENOMYCIN

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Amiclenomycin (AM) was found to be a strong inhibitor of KAPA*-DAPA* aminotransferase of *Brevibacterium divaricatum*. This transamination was suggested to follow Ping Pong Bi Bi mechanism. Inhibition of this transamination by AM is of a non-competitive type in a LINEWEAVER-BURK plot of initial velocity, but not in a DIXON plot. The activity of KAPA-DAPA aminotransferase drops abruptly after preincubation with AM, but its activity is restored by dialysis against 10 mM potassium phosphate buffer (pH 7.0). Inhibition by AM is decreased by an increase of KAPA in the reaction mixture, but not by an increase of S-adenosyl-L-methionine (SAM) or pyridoxal-5′-phosphate (PALP). These facts indicate that AM exerts its inhibitory action against KAPA-DAPA aminotransferase by binding to the enzyme, probably to the KAPA-DAPA binding site.

As reported in our previous paper[1], amiclenomycin was thought to inhibit the KAPA-DAPA transamination reaction because KAPA accumulation increased in the presence of AM and the action of AM was reversed by addition of biotin biosynthesis intermediates such as DAPA and desthiobiotin (DTB). The present paper deals with the mode of inhibition of KAPA-DAPA aminotransferase by AM.

Materials and Methods

Organisms. *Brevibacterium divaricatum* NRRL 2311, *Pseudomonas graveolens* IFO 3460 and *Bacillus subtilis* AKU 0236 were obtained through the courtesy of Prof. K. OGATA, Kyoto University.

Preparation of enzymes. KAPA-DAPA aminotransferase was prepared from *Brev. divaricatum* according to the method of IZUMI *et al.*[2] Cells grown at 27°C for 24 hours were washed, disruped in a French press (1,200 kg/cm^2) and the enzyme was partially purified (*ca* 16 times in specific activity) by ammonium sulfate fractionation (50% saturation) and DEAE-cellulose column chromatography. The enzyme was eluted from the column with a gradient from 50 to 250 mM of potassium phosphate buffer (pH 7.0) containing 5 mM of mercaptoethanol. Active fractions were concentrated by the addition of ammonium sulfate to 60% saturation and dialyzed against a continuous flow of 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM of mercaptoethanol. The enzyme was used within 2~3 weeks after preparation.

Ureido ring synthetase which converts DAPA to DTB was prepared from *Ps. graveolens* by the method of OGATA *et al.*[3,4] The enzyme was partially purified (*ca* 16 times in specific activity) by ammonium sulfate fractionation (20~50% saturation) and DEAE-cellulose column chromatography. This enzyme preparation contained no KAPA-DAPA aminotransferase activity and was stable for at least 2 months at 4°C.

Enzyme assays. The method of OGATA (personal communication) was employed for KAPA-DAPA transamination. The basal reaction mixture (0.5 ml) contained KAPA 5 nmoles, SAM 500 nmoles, PALP 50 nmoles, potassium phosphate buffer (pH 8.0) 50 μmoles and KAPA-DAPA

* KAPA: 7-Keto-8-aminopelargonic acid. DAPA: 7, 8-Diaminopelargonic acid.

aminotransferase, unless otherwise described. The reaction was carried out at 37°C and arrested by heating the mixture for 2 minutes in a boiling water bath. The DAPA formed was determined by bioassay after conversion to DTB. The reaction mixture arrested was incubated at 37°C for 2 hours with the ureido ring synthetase system (0.5 ml) as described below. DTP thus converted from DAPA was bioassayed by a paper disc method with *B. subtilis*[5]. A combined reaction mixture (1 ml) of KAPA-DAPA aminotransferase and ureido ring synthetase was also employed according to the method of IZUMI *et al.*[2]

The ureido ring synthetase system contained $NaHCO_3$ 50 μmoles, ATP disodium salt 10 μmoles, $MgCl_2$ 10 μmoles and ureido ring synthetase 2.5 units in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.2)[4]

Chemicals. KAPA·HCl was a kind gift of Ajinomoto Co. Inc.

Results

Effect of Amiclenomycin on the Activity of KAPA-DAPA Aminotransferase

Since the assay of KAPA-DAPA aminotransferase activity employed involves two reactions as described in Materials and Methods, the effect of AM on ureido ring synthetase was first examined.

Table 1 shows, however, that AM exhibited no significant activity against ureido ring synthetase even when AM was added at a concentration 40 times higher than that of DAPA. Therefore, it was confirmed that this assay can be used for the determination of KAPA-DAPA aminotransferase activity.

As expected from the previous paper[1], AM inhibited KAPA-DAPA aminotransferase.

Table 1. Effect of amiclenomycin on ureido ring synthetase

DAPA (nmole/ml)	AM (nmole/ml)	DTB formed (nmole/ml)	Inhibition (%)
2.5	0	0.45	—
	25	0.44	2.3
	100	0.42	6.6
5.0	0	0.67	—
	25	0.67	0
	100	0.64	4.5
10.0	0	0.79	—
	25	0.79	0
	100	0.82	0
20.0	0	1.07	—
	25	1.07	0
	100	1.07	0

Incubation time was 60 minutes

Fig. 1. Inhibitory effect of amiclenomycin on KAPA-DAPA aminotransferase.

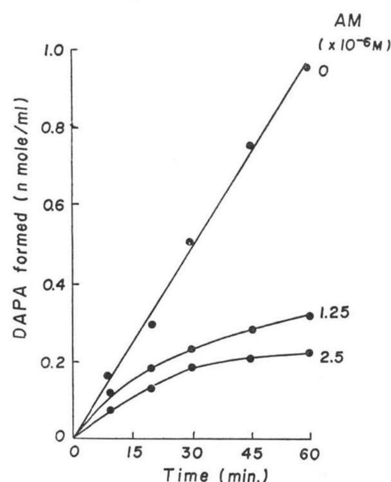
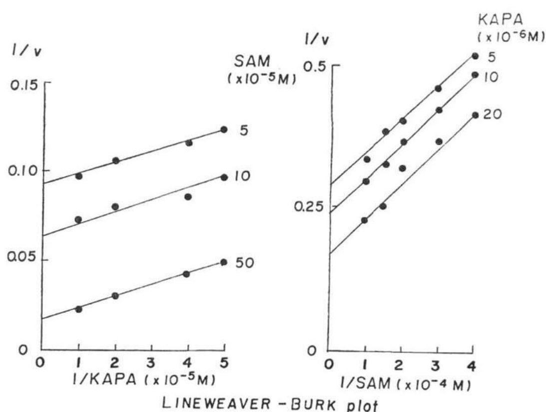


Fig. 2. Initial velocity patterns for the forward reaction



The time course of the reaction with or without addition of AM is shown in Fig. 1. A linear reaction curve was obtained until 60 minutes when AM was not added. The rate of inhibition by AM was not constant with reaction time and was higher at prolonged reaction times. The reaction curves with AM reached a plateau with time suggesting that KAPA-DAPA aminotransferase is gradually inactivated by AM.

Mechanism of KAPA-DAPA Transamination

KAPA-DAPA transamination is a unique transamination reaction because SAM serves as the amino donor. The mechanism, however, has not yet been clarified, though it has been known that the amino acceptor (KAPA) at high concentration exhibits substrate inhibition in this reaction[6]. In order to clarify the mode of inhibition by AM, it was necessary to study the mechanism of KAPA-DAPA transamination.

Fig. 2 shows initial velocity patterns for the reaction leading to the formation of DAPA using different concentrations of either KAPA or SAM. Double reciprocal plots of reaction velocities against concentrations of one substrate at three different fixed levels of the second substrate gave parallel lines. Replots of intercepts versus reciprocal concentrations of the fixed substrate were linear. These initial velocity patterns indicate that kinetics of transamination by KAPA-DAPA aminotransferase obey the initial velocity equation of Ping Pong Bi Bi mechanism[7]:

$$\frac{1}{v}=\frac{1}{V_{\max}}\left(\frac{Ka}{A}+\frac{Kb}{B}+1\right),$$

where A and B are concentrations of two substrates and Ka and Kb are their dissociation constants. Initial velocity studies for the backward reaction (from DAPA to KAPA) could not be performed because deaminated SAM was not available as a substrate.

We confirmed the observation by PAI[6] that KAPA at high concentration shows substrate inhibition (Fig. 3). This type of curves is known to appear in the substrate inhibition of enzyme reactions following Ping Pong mechanism[8]. This inhibition has been understood to be due to the binding of the second substrate to the enzyme E as well as the enzyme NH_2-E shown in Fig. 4.

Thus, we feel that KAPA-DAPA transamination proceeds by the Ping Pong Bi Bi mechanism as in other transamination reactions[7,9,10,11].

Initial Velocity Pattern in the Presence of Amiclenomycin

Initial velocity of KAPA-DAPA transamination in the presence of AM was meas-

Fig. 3. Competitive substrate inhibition by KAPA in KAPA-DAPA transamination

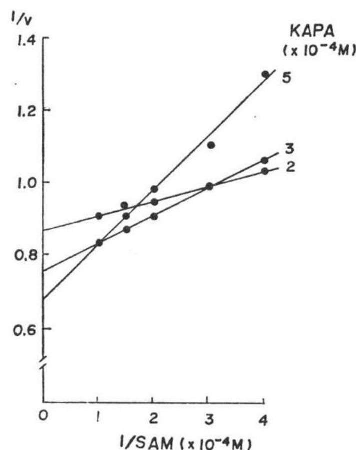


Fig. 4. Proposed mechanism of KAPA-DAPA transamination

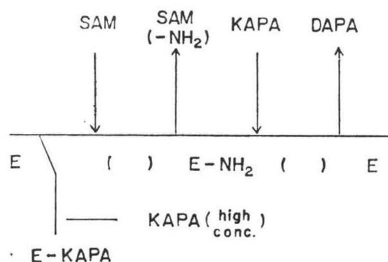
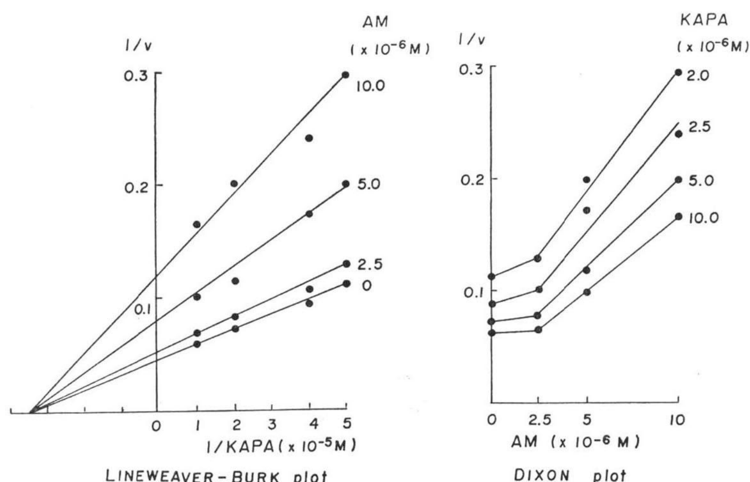


Fig. 5. Inhibition pattern with amiclenomycin against KAPA-DAPA aminotransferase reaction

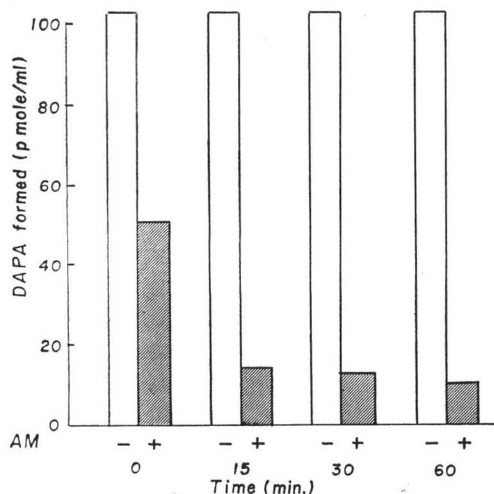


ured by incubating the reaction mixture for 20 minutes. Fig. 5 shows a LINEWEAVER-BURK plot and DIXON plot with KAPA. Double reciprocal plots in LINEWEAVER-BURK plot showed apparently noncompetitive inhibition, but a certain curvature was obtained in DIXON plot. Similar results were obtained with SAM as varied substrate. This inhibition pattern can be explained as a mutual depletion system[12] and may indicate that KAPA-DAPA aminotransferase is inactivated by tight-binding with AM.

Fig. 6. Effect of preincubation of amiclenomycin with KAPA-DAPA aminotransferase

One ml of KAPA-DAPA aminotransferase solution (*ca.* 2 units*/ml) was preincubated at 37°C with 1 ml of amiclenomycin solution (25 nmoles/ml), then 0.2 ml of the mixture was added to 0.3 ml of basal reaction mixture and incubated for 30 minutes at 37°C.

* One unit was defined as the amount of enzyme necessary to synthesize 1 nmole of DAPA under the assay condition



Tight Binding of Amiclenomycin to KAPA-DAPA Aminotransferase

If AM binds tightly or irreversibly to KAPA-DAPA aminotransferase, preincubation of the enzyme with AM should result in increase of the inhibition by AM of the enzyme activity. This is shown in Fig. 6. The enzymic activity dropped abruptly 15 minutes after mixing with AM and thereafter the activity decreased gradually to less than 10 % of the control (no addition of AM).

However, a recovery of the enzymic activity from the inactivated form was clearly observed by dialysis against 10 mM potassium phoshate buffer (pH 7.0) containing 5 mM mercaptoethanol. The enzymic activity after dialysis was 55.2 % of that of the control while the activity without dialysis remained at the inactivated level (9.1 %) of the control as indicated in Table 2.

Therefore, we conclude that AM does not bind to KAPA-DAPA aminotransferase irreversibly.

Table 2. Recovery of enzyme activity by dialysis of inactivated KAPA-DAPA aminotransferase caused by amiclenomycin

AM	Dialysis	DAPA formed (pmoles/ml·15′)	Activity (%)	Recovery (%)
−	−	420	100.0	
+	−	38	9.1	
+	+	232	55.2	46.1

KAPA-DAPA aminotransferase solution (*ca* 20 units/ml) was preincubated with equal volume of amiclenomycin solution (50 nmoles/ml) and then 1 ml of the mixture was dialyzed against a continuous flow of 10 liters of potassium phosphate buffer (10 mM, pH 7.0) at 4°C for 20 hours.

Effect of KAPA on the Action of Amiclenomycin

Since the structure of AM resembles that of KAPA and the antibiotic needs a considerable time to bind to KAPA-DAPA aminotransferase completely, inhibition of KAPA-DAPA aminotransferase by AM was examined in the presence of increased amounts of KAPA. Effect of SAM or PALP was also examined.

Inhibition by AM (5 nmoles/ml) was decreased in proportion to the increasing amounts of KAPA (10∼200 nmoles/ml); activity of KAPA-DAPA aminotransferase was improved from 46.4% to 77.4% of the control without AM by increasing the concentration of KAPA from 10 nmoles/ml to 200 nmoles/ml (Table 2). On the other hand, increased amounts of SAM exhibited a tendency to enhance inhibition by AM of KAPA-DAPA aminotransferase activity. PALP did not lower the activity of AM. These results can be explained if AM exerts its inhibitory action against KAPA-DAPA aminotransferase by occupying the KAPA-binding site of the enzyme.

In this connection, it should be noted that AM is more acidic and more basic than KAPA:

Table 3. Effect of KAPA on the action of amiclenomycin

KAPA (nmole/ml)	SAM (nmole/ml)	PALP (nmole/ml)	DAPA formed (pmole/ml)		Inhibition (%)
			AM* −	AM* +	
10	500	50	140	65	53.6
50	500	50	140	77	45.0
100	500	50	140	103	26.4
200	500	50	124	96	22.6
10	50	50	84	51	39.3
10	100	50	89	47	47.2
10	250	50	103	56	45.6
10	500	500	127	60	52.8

* AM was added in concentration of 5 nmoles/ml and reaction mixtures were incubated for 60 minutes.

Table 4. Effects of aromatized amiclenomycin and γ-phenylbutyrine on the activity of KAPA-DAPA aminotransferase

	nmole/ml	DAPA formed (pmole/ml)	Activity (%)
Control	0	237	100.0
Arom.AM*	5	225	94.9
	25	225	94.9
	50	225	94.9
PB*	5	210	88.6
	25	210	88.6
	50	189	79.7

Incubation time was 30 minutes.
* Arom.AM: Aromatized amiclenomycin
PB: γ-Phenylbutyrine

pKa′ 2.0 (carboxylic group), 8.7 (α-amino group) and 9.9 (4′-amino group) in AM[13] and pKa′ 4.6 (carboxylic group) and 8.6 (8-amino group) in KAPA. These stronger functional groups in AM may cause tighter binding to KAPA-DAPA aminotransferase.

Effect of Amiclenomycin Derivative on KAPA-DAPA Aminotransferase

Amiclenomycin derivatives such as aromatized amiclenomycin and γ-phenylbutyrin have no antimicrobial activity even against organisms susceptible to AM. This may be due to the inactivity against KAPA-DAPA aminotransferase or to the inability to permeate through membrane. These derivatives exhibited only a weak inhibitory activity against the enzyme when 50 nmoles/ml were added, as shown in Table 3.

Discussion

It is shown in this paper that amiclenomycin binds tightly to KAPA-DAPA aminotransferase, probably to the KAPA binding site, and inhibits this enzyme reaction. AM is the first natural inhibitor shown to act on KAPA-DAPA transamination in biotin biosynthesis and so it should be useful as a biochemical tool for investigation of biotin biosynthesis in organisms.

MSD 235 S was reported to inhibit the growth of *Escherichia coli* in a biotin-free medium and to be reversed by biotin[14,15,16]. BAGGALEY *et al.*[17] determined its structure which is a dipeptide containing AM moiety and named it stravidin. They noted a structural correlation between KAPA and stravidin, but have not yet elucidated its mode of action. However, in view of our data, stravidin probably exerts its activity through its AM moiety.

Amiclenomycin derivatives such as aromatized AM and γ-phenylbutyrine showed a very weak inhibitory activity against the enzyme in comparison with AM. The boat form of the 2, 5-cyclohexadiene ring appears to be important for the inhibitory action of AM against KAPA-DAPA aminotransferase, since a conformational difference between AM and aromatized AM is present only in the six-membered ring. Moreover, aromatization should weaken the basicity of the 4′-amino group and may lower the affinity for the enzyme.

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