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Kinetics of Intracellular Adenosine Deaminase to Substrate Analogs and Inhibitors in *Aspergillus oryzae*

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Kinetic parameters of various substrates and inhibitors were measured to elucidate the binding requirements of the active site of intracellular adenosine deaminase (ADA) in *Aspergillus oryzae*. 3′-Deoxyadenosine was the best substrate according to the value of relative kcat/K_m. Purine riboside was found to be the strongest inhibitor with the K_i value of 3.7×10^{-5} M. Adenine acted neither as a substrate nor as an inhibitor, suggesting the presence of ribose at N-9 of adenosine was crucial to binding. ADA also catalyzed the dechlorination of 6-chloropurine riboside, generating inosine and chloride ions. Substrate specificity of 6-chloropurine riboside was 0.86% of adenosine. Purine riboside, a competitive inhibitor of ADA, inhibit the dechlorination with similar K_i value, suggesting that the same binding site was involved in deamination and dechlorination. Among the sulfhydryl group reagents, mercurials, p-chloromercuribenzoate (PCMB), mersalyl acid and $HgCl_2$ inactivated the enzyme. Mersalyl acid-inactivated ADA was reactivated by thiol reagents, but PCMB-inactivated enzyme was not. When ADA was treated with the mercurial reagents, the inhibition constants and inhibition patterns were determined. Each inhibition was competitive with substrate. The K_i values of these mercurial reagents were lower in 10 mM phosphate buffer than in 100 mM phosphate buffer, showing phosphate dependency.

KEY WORDS □ adenosine deaminase, intracellular enzyme of *Aspergillus oryzae*, adenosine, 6-chloropurine riboside, 3′-deoxyadenosine, purine riboside, SH group

Adenosine and 2′-deoxyadenosine are purine nucleosides that are intermediates in the pathway of purine nucleotide degradation. Many biological properties have been identified; it is toxic to mammalian and bacterial cells and its presence is associated with inhibition of the immune system and changes in the various metabolism (10). The turnover of adenosine and 2′-deoxyadenosine in cells requires a complicated series of reactions and results in low or unmeasurable amount of adenosine under normal conditions. Adenosine deaminase (ADA) catalyzes the irreversible hydrolytic deamination of adenosine and its analogs to corresponding inosine and ammonia. ADA has a wide spread distribution in animal tissues (1, 9, 12, 16) and marine invertebrates (4, 23, 27). Several microorganisms have been demonstrated for the source of the enzyme (5, 11, 22, 28, 29). A number of pure enzymes have been obtained from *Pseudomonas iodinum* (26), *Klebsiella* sp. (15), *Micrococcus sodonensis* (25), *Saccharomyces cerevisiae* (18), extracellular source of *Streptomyces* sp. (13) and Takadiastase from *Aspergillus oryzae* (20). Intracellular ADA in *Aspergillus oryzae* has been purified recently and partially characterized (7). The purified enzyme was specific for adenosine, 2′-deoxyadenosine and several adenosine analogs.

In this work, kinetic parameters of various substrates and inhibitors were measured to investigate the binding requirements of the active site of intracellular ADA in *Aspergillus oryzae*. We reported on the nature of dechloronase activity as catalyzed by ADA. In addition, inhibitory effects of mercurial agents such as PCMB, mersalyl acid and $HgCl_2$ on the enzyme were observed to elucidate the involvement of sulfhydryl group in the active site of enzyme.

MATERIALS AND METHODS

Enzyme purification

Aspergillus oryzae KCTC 2114 was used for the preparation of the enzyme, adenosine deaminase. All purification procedures were followed as described in the previous paper (7).

Determinations of kinetic parameters

The deamination of adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 2′-adenosine monophosphate, 3′-adenosine monophosphate, 5′-adenos-

ine monophosphate, 2′,3′-o-isopropylidene adenosine, 5′-deoxy-5′-iodoadenosine (5′-IAdo), 5′-deoxy-5′-thioisobutyladenosine (SIBA), 5′-deoxy-5′-methylthioadenosine (MTA), and adenine 9-B-arabinofuranoside were measured by a spectrophotometric assay. The assay was based on the measurement of the decrease in absorbance at 265 nm resulting from the conversion of adenosine moiety of the above compounds to inosine ($\Delta E = -8.6 \times 10^3\ M^{-1}cm^{-1}$) (1). The deamination of formycin A was estimated by the decrease of absorbance at 305 nm ($\Delta E = -6 \times 10^3\ M^{-1}cm^{-1}$). The dechlor ination of 6-chloropurine riboside was tested at 250 nm by the increase of absorbance due to generation of inosine ($\Delta E = 5.6 \times 10^3\ M^{-1}cm^{-1}$). Spectrophotometer (Kontron, UVKON 860) equipped with RS 232C-interface was used. The reaction mixture contained, in a final volume of 1 m*l*, 50 mM Tris-Cl, pH 7.2 and various concentrations of adenosine in the presence or absence of inhibitor All reaction mixture except ADA was preincubated for 3 min. The reaction was initiated by the addition of enzyme and continued for about 2 min to get the linear region. The values of K_m and V_m were calculated from Lineweaver-Burk plot. Reciprocal of velocities were plotted graphically versus reciprocal of substrate concentrations. Curves were fitted to equation

$$1/v = K_m/V_m \cdot 1/S + 1/V_m$$

The K_i values of inhibitors were obtained in the replot of inhibitor concentrations versus slopes from Lineweaver-Burk plot or in Dixon plot. Points on the kinetic curves represented average of two or three determinations, but in the calculations, each determination was treated individually.

RESULTS

Substrate specificity

It was demonstrated in the previous paper (7) that intracellular ADA in *Aspergillus oryzae* was capable of catalyzing the deamination of adenosine, adenine 9-B-arabinofuranoside, formycin A, 2′-adenosine monophosphate (AMP), 3′-AMP, 5′-AMP, 2′-deoxyadenosine, 3′-deoxyadenosine, 6-chloropurine riboside (6-CPR) and 2′,3′-o-isopropylidene adenosine. We attempted to determine the Michaelis constants and reaction velocities of the compounds to get the informations for substrate specificity. Table 1 presented kinetic parameters of adenosine and a number of purine nucleoside analog with purified fungal ADA. Fig. 1 showed the structures of some of these compounds. Among the compounds included are those with modifications in the structure of the purine ring, in the substituents on the purine ring and in the carbohydrate moiety. Alterations in the purine ring resulted in significant decrease of substrate activity. The compounds, formycin A and 6-CPR were deaminated about 0.058 and 0.167-fold slower than adenosine, respectively. Other compounds which were modified on the purine ring, N^6-methyladenosine, N^6,N^6-dimethyladenosine, purine riboside and 6-methylpurine riboside were devoid of substrate activity. The V_m of 3′-deoxyadenosine was 2-fold higher and the K_m was about 2-fold lower than those of adenosine, suggesting that 3′-deoxyadenosine had best substrate activity among tested compounds. Changes in C-5′ position resulted in not so much effect on reaction velocities and increased the affinities of compounds except SIBA, probably due to the steric effect. By calculating the values of relative kcat/K_m, the order of substrate specificity was determined to be 3′-deoxyadenosine, 5′-IAdo, MTA, 5′-AMP, adenosine, 2′,3′-o-isopropylidene adenosine, SIBA, adenine 9-B-arabinofuranoside, 2′-deoxyadenosine, 3′-AMP, 2′-AMP, formycine A and 6-CPR. The compounds, 3′-deoxyadenosine, 5′-IAdo, MTA and 5′-AMP were found to be better substrates than adenosine.

Table 1. *Kinetic parameters of various substrates for adenosine deaminase.*

Compound	K_m (μM)	Relative V_m	Relative kcat/K_m
Adenosine	455	100	1
Formycin A	118	5.8	0.037
6-CPR	1250	16.6	0.0086
2′-dAdo	714	59.9	0.42
3′-dAdo	200	225	5.1
2′-AMP	85	10.8	0.19
3′-AMP	112	20.3	0.27
5′-AMP	196	67.1	1.58
2′,3′-O-isopropylidine Ado	200	41.9	0.95
5′-IAdo	222	85.6	1.93
SIBA	667	92.8	0.69
MTA	264	92.8	1.72
Adenine 9-B-arabinofuranoside	303	35	0.53

Inhibitors of ADA

In order to investigate the structure and activity relationship further, several nucleosides were treated as inhibitors when adenosine was a substrate. As shown in Table 2, the inhibition constants (K_i) and inhibition patterns for several adenosine analogs were estimated from replot of double reciprocal plot or Dixon plot. Of the analogs presented, purine riboside (6, 14, 24) was a relatively potent competitive inhibitor with K_i value of 37 μM. The affinity of formycin A as an inhibitor (K_i, 95 μM) was similar to that as a

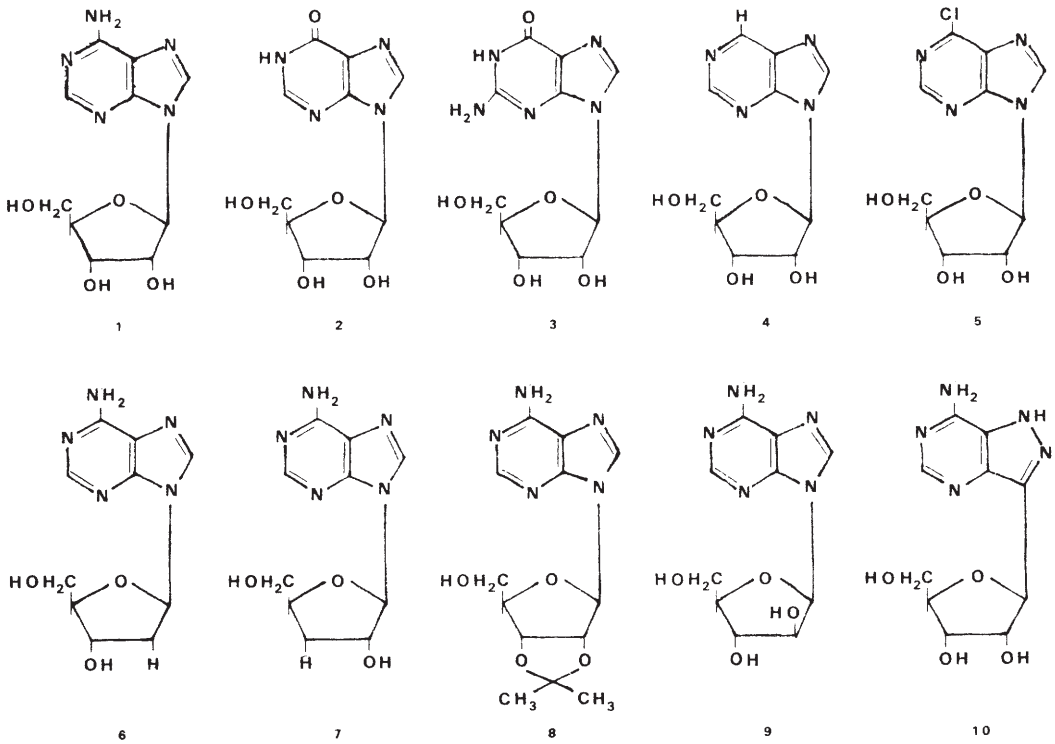

Fig. 1. *Structure of adenosine and adenosine analogs.*
1, adenosine; 2, inosine; 3, guanosine; 4, purine riboside; 5, 6-chloropurine riboside; 6, 2′-deoxyadenosine; 7, 3′-deoxyadenosine; 8, 2′,3′-o-isopropylidene adenosine; 9, adenine 9-B-arabinofuranoside; 10, formycin A.

Table 2. *K_i values and inhibition patterns of substrate analogs for adenosine deaminase.*

Compounds	K_i (μM)	Inhibition pattern
Purine riboside	37 (41)	competitive
N^6-Methyladenosine	400 (400)	non-competitive
N^6,N^6-dimethyladenosine	465 (435)	non-competitive
8-Aminoguanosine	530 (530)	non-competitive
Formycin A	− (95)	non-competitive

The K_i values were obtained from replots of double reciprocal plots and the values in paranthesis were from Dixon plots. All the values were average of 2/3 observations.
−, no determination.

substrate (K_m, 118 μM). No substrate activity occurred with N^6-methyladenosine and N^6,N^6-dimethyladenosine, but those acted as inhibitors with similar binding capacity to normal substrate, adenosine. 6-Mercaptopurine riboside acted neither as an inhibitor nor as a substrate. A number of other compounds failed to act as inhibitors under the assayed conditions. These were inosine, 2′-deoxyinosine, formycin B, hypoxanthine, guanine, guanosine, adenine, cytosine and cytidine.

Dechloronase activity

Adenosine deaminase catalyzed the dechlorination of 6-CPR generating, as products of the reaction, inosine and chloride ions. The kinetic parameters were compared for adenosine and 6-CPR in Table 1. The Michaelis constant for the 6-CPR is 1.25×10^{-3} M as compared with 4.6×10^{-4} M for adenosine. The K_m value of 6-CPR is approximately three times greater than that of adenosine, while the V_m is only one fourth as large as that of adenosine. The substrate specificity of adenosine for the enzyme was about 117 times higher than that of 6-CPR. Purine riboside, which have been shown to competitively inhibit ADA, also inhibited the dechlorination reaction. The inhibition constants, K_i values were estimated to be 47 μM and 50 μM for dechlorination and deamination, respectively. The effect of temperature on stability of the enzyme for dechlorination was very similar to that for deamination as determined previously (7). When the enzyme was incubated at 55 and 60°C for 1 hr, the enzyme was reasonably stable at 55°C, but rapidly inactivated at 60°C. The dechlorination of 6-CPR (5×10^{-4} M) was inhibited 87%

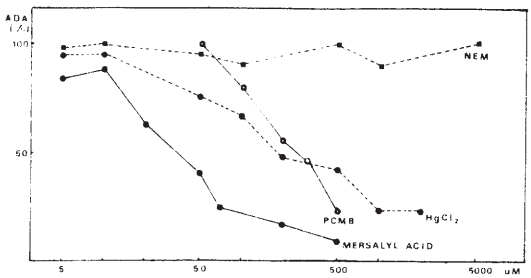

Fig. 2. *Inactivation of adenosine deaminase by different concentrations of PCMB, mersalyl acid, $HgCl_2$ and N-ethylmaleimide.*
The reaction mixture contained 10 mM KPO_4, pH 7.2, various concentrations of inhibitor and adenosine deaminase and was incubated at room temperature for 40 min (PCMB) or 30 min (others). Aliquots of 30 μl were assayed with 100 μM adenosine and 10 mM KPO_4, pH 7.2.

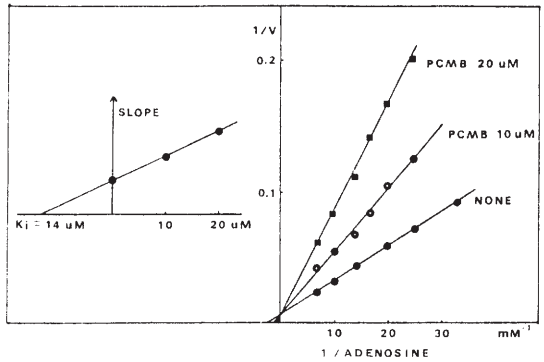

Fig. 3. *Inhibition of adenosine deaminase by PCMB in 10 mM phosphate.*
Plots of reciprocal of initial velocity with respect to reciprocal of adenosine concentration. The inhibition constant, K_i of PCMB as the competitive inhibitor of adenosine, was estimated to be 1.4×10^{-5} M by the replot shown in the inset.

in the presence of adenosine (2×10^{-4} M). The deamination of adenosine (1.5×10^{-4} M) was inhibited 85% in the presence of 6-CPR (3×10^{-4} M). The initial rates were much lower for 6-CPR. The reaction velocity of 6-CPR (5×10^{-4} M) was 2% of that of adenosine (1.5×10^{-4} M). The value of total velocity of the reactions lay between each of two reactions. These results suggested that a single enzyme catalyzed two separate reacions. The reversibility of deamination reaction was tested. When inosine, chloride ions and the substantial amounts of ADA were incubated at 30°C, no spectral change from 220 to 300 nm was observed up to 150 min of incubation period, indicating that 6-CPR was not formed.

Effects of mercurial reagents

Deamination of adenosine by ADA was found to be inhibited by mercuric ion and a variety of organic mercurials as described in the previous paper (7). When the enzyme was incubated at room temperature with p-chloromercuribenzoate (PCMB) or mersalyl acid, no further change of inactivation was observed after 20 min for PCMB and abruptly for mersalyl acid. Fig. 2 showed the inactivation of ADA when the enzyme was treated with various concentrations of PCMB or mersalyl acid. ADA was also incubated for 30 min with various concentrations of $HgCl_2$ and N-ethylmaleimide (NEM). The enzyme was inhibited through the broad concentration range of $HgCl_2$, but NEM showed no effect on ADA in the concentration range of 5 to 5000 μM. It indicated that NEM reacted with different sulfhydryl groups from others, such as PCMB, mersalyl acid and $HgCl_2$ and these were not related to active site of enzyme. It was also possible that NEM did not bind the enzyme. The effect of thiol reagents on the enzyme inactivated by mercurial agents was observed. When the enzyme was treated with 500 μM of mersalyl acid, complete inactivation of enzyme occurred. The enzyme regained the full activity after 30 sec incubation with excess of thiol reagent (results not shown). The thiol reagents, which recovered inactivation of ADA, were dithiothreitol (DTT), 2-mercaptoethanol, glutathione and cysteine. The inactivation by mersalyl acid was reversed completely by the addition of the thiol reagents, suggesting that ADA was not irreversibly inactivated by mersalyl acid. The other mercurial agent, PCMB was treated with the concentration of 500 μM. After the total inactivation occurred, no thiol reagent reactivated the enzyme. In contrast to mersalyl acid, the inactivation of enzyme by PCMB seemed not to be reversed by any tested thiol reagent. When the enzyme was treated with the mercurial reagents,

Table 3. *K_i values and inhibition pattern for adenosine deaminase by mercurial reagents in potassium phosphate buffers.*

Inhibitor	10 mM phosphate	100 mM phosphate
PCMB	1.3×10^{-5} M competitive	1.5×10^{-4} M competitive
Mersalyl acid	1.4×10^{-6} M competitive	1.5×10^{-5} M competitive
$HgCl_2$	5.4×10^{-5} M competitive	1.4×10^{-4} M competitive

the inhibition constants and inhibition patterns were determined. In each case, inhibition was competitive with the substrate. Fig. 3 showed competitive inhibition of ADA by PCMB. Table 3 presented inhibition constants obtained for PCMB, mersalyl acid and $HgCl_2$ in potassium phosphate buffers, pH 7.2. The values of inhibition constants for these mercurial reagents were lower about one magnitude in 10 mM phosphate buffer than in 100 mM phosphate buffer, showing phosphate ion dependency.

DISCUSSIONS

Adenine acted neither as a substrate nor as an inhibitor to intracellular ADA in *Aspergillus oryzae*. These results suggested that the presence of ribose at N-9 of adenosine was crucial to binding of the compound to the enzyme. The pentose moiety of adenosine appeared to play a role of positioning the purine base moiety of the substrate to the active site. The existance of ribose at N-9 was reported to be important for transition state formation in the enzyme and substrate complex in mussel and calf intestine enzyme (23, 31). Both of K_m and relative V_m values for several adenosine derivatives were apparently affected in various degrees according to the position of substitution. When the hydroxyl group of adenosine was replaced by a hydrogen at C′-2, the increase of K_m value was observed. On the other hand, inversion of the hydroxyl group at C′-2, as in arabinosyl adenine, resulted in the decrease of K_m value. The value of K_m of 3′-deoxyadenosine and 2′,3′-o-isopropylidene adenosine were about half of the value of adenosine. These results indicated that the hydroxyl group at C′-3 interfered the binding of substrate and the inversion of the hydroxyl group at C′-2 on the furanosyl ring increased the affinity. There have been examinations in details of the role of the substituents at C-2′ and C-3′ (1, 4, 6, 19, 21). When the hydroxyl group was replaced by phosphate at C′-2 or C′-3, the affinity increased significantly. Substituted analogs at C′-5 showed better affinity than adenosine except SIBA. The replacement of the hydroxyl group at C′-5 by bulky groups such as iodine atom, methylthio and phosphate groups seemed to allow the distance enough to react the binding site of the enzyme, if it was not too big like thioisobutyl group. The relative V_m values of 2′-, 3′-deoxyadenosine and 2′,3′-o-isopropylidene adenosine was different from that of adenosine. Change in C′-3 accelerated the reaction, but that in C′-2 deccelerated. Replacement of the hydroxyl by phosphate group at C′-2 or C′-3 position significantly lowered the activity for adenine deamination and that at C′-5 slightly lowered. The bulky groups at C′-5 showed no effect on the reaction velocity. These data emphasized the importance of the 9-position substituent in forming the complex of enzyme and substrate. It seemed that the binding of substrate to the active site and the final orientation for reaction were controlled by the pentose moiety of nucleoside.

Replacement of C-8 by a nitrogen atom and N-9 by a carbon atom as seen in formycin A markedly enhanced the affinity (K_m and K_i values of formycin A, 118 μM and 95 μM, respectively), but decreased the deamination rate significantly.

The addition of methyl group to amino group at C-6 showed no change of affinities in N^6-methyladenosine and N^6,N^6-dimethyladenosine. This suggested that steric factor of amino group at C-6 did not affect the binding of the compound to the enzyme. The replacement of C-6 by sulfur atom did not render the compound to bind in 6-mercaptopurine riboside, but the affinity of purine riboside, which was deaminated compound at C-6, was improved significantly. Purine riboside and 6-mercaptopurine riboside have been reported to be competitive inhibitors with bovine and chicken enzyme (14, 17). When keto group was substituted for amino group at C-6, no binding was observed in the case of inosine or guanosine. These results indicated that the position of C-6 was part of important binding site to the enzyme.

Replacement of a chlorine atom for amino group at C-6 made a nucleoside act as a substrate for the enzyme. Dechlorination of 6-chloropurine riboside was catalyzed by Takadiastase ADA, calf intestine and mussel enzyme (8, 20, 23). When the V_m values for dechlorination were compared with that for deamination, Takadiastase enzyme showed similar values for dechlorination and deamination (20). The relative V_m value for 6-chloropurine riboside was 17% of deamination of adenosine in intracellular ADA in *Aspergillus oryzae*. The ratios of 25% and 14% were estimated in calf intestinal and mussel ADA, respectively, implying that the enzyme acted in a similar pattern to these enzymes (8, 23). The dechlorination products were inosine which was detected by spectral change and chloride ion. The Michaelis constant of 6-chloropurine riboside was about 6 times greater than that of 3′-deoxyadenosine, best substrate among the tested compounds and relative velocity was only 7% of that of 3′-deoxyadenosine. Based on the substrate specificity, 3′-deoxyadenosine and adenosine were about 600 and 116 times better substrates, respectively than 6-chloropurine riboside. Purine riboside was a competitive inhibitor for deamination and dechlorination with K_i values of 50 and 47 μM, respectively. These results suggested that the same binding site of the enzyme was involved in deamination and dechlorination.

It has been reported that PCMB inhibited ADA activity of calf spleen, mussel and clam ADA. The

competitive type of PCMB inhibition was found in Takadiastase ADA, clam and mussel ADA (2, 3, 30). The similar phenomena was observed for intracellular ADA in *Aspergillus oryzae*. These results suggested that sulfhydryl groups could be responsible for the substrate binding site in this type of enzymes. When adenosine was used as a substrate, the inhibition constant of PCMB was 1.4×10^{-5} M in 10 mM phosphate buffer and 1.5×10^{-4} M in 100 mM phosphate buffer. The K_i values of mersalyl acid and $HgCl_2$ also showed about one magnitude difference between 10 and 100 mM of phosphate concentrations of assay conditions. Although phosphate ion did not act as a substrate, the K_i values were different due to concentration of phosphate. The effect of phosphate ion on PCMB inhibition of intracellular ADA in *Aspergillus oryzae* was different from mussel ADA, but similar to that of clam ADA (2, 3). The repression of PCMB inhibition by addition of phosphate could be contributed by the capability of phosphate ion to dissociate enzyme-inhibitor complex, resulting in the increase of K_i values. The sulfhydryl group, which located near active site of enzyme, appeared to be protected against PCMB by phosphate ions. The orientation of the functional sulfhydryl groups into the catalytically correct position might be caused by phosphate ions. Competitive inhibition by all tested mercurial agents indicated that mercury atom was responsible for inhibition and the binding of substrates and mercurials was mutually exclusive. These inhibitions suggested that the sulfhydryl groups of the enzyme were involved in binding of inhibitors. Inactivated enzyme by PCMB was not reactivated, while that by mersalyl acid was fully regained the activity. The reversibility of mersalyl acid-inactivated ADA by DTT suggested that the competitive inhibition by mersalyl acid was generated by blocking of the catalytic sulfhydryl group without large scale of change in active site of enzyme. PCMB and mersalyl acid seemed to bind different reactive sulfhydryl groups of the enzyme.

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초 록: *Aspergillus oryzae*의 세포내 효소인 Adenosine Deaminase의 기질 유사체와 억제물질에 대한 반응속도론적 분석

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Adenosine deaminase(ADA)의 여러 기질과 억제물질의 반응 속도론적 상수가 *Aspergillus oryzae*의 세포내 효소인 ADA의 활성자리에 어떻게 부착하고 어떤 요인을 필요로 하는지를 알기위해 측정되어졌다. Relative kcat/K_m값에 의하면 조사된 기질로 작용하는 화합물 중에 3′-deoxyadenosine이 가장 좋은 기질로 작용하는 것으로 밝혀졌다. 몇 개의 유사체가 억제물질로 조사되었는데 purine riboside가 3.7×10^{-5} M의 K_i값을 가지고 가장 강한 억제물질로 나타났다. Adenine은 기질로도 억제물질로도 작용을 못하므로 adenosine의 N-9 위치의 ribose가 효소에 부착하는데 중요하다는 것을 시사하고 있다. 또 ADA는 6-chloropurine riboside(6-CPR)의 dechlorination을 촉매화하여 inosine과 Cl이온을 생성한다. 6-CPR의 ADA에 대한 기질 특이성은 정상 기질인 adenosine의 0.86%로 측정되었다. ADA의 경쟁적 억제물질인 purine riboside는 비슷한 K_i값을 가지고 dechlorination도 억제하므로 deamination과 dechlorination 반응은 효소의 부착자리를 공유하고 있다고 생각되어진다. SH기에 작용하는 화합물중 수은제인 p-chloromercuribenzoate(PCMB), mersalyl acid, $HgCl_2$는 효소의 deamination 반응을 억제했다. Mersalyl acid에 의해 활성이 억제된 ADA는 thiol reagent인 dithiothreitol이나 2-mercaptoethanol에 의해 활성이 회복되지만 PCMB에 의해 억제된 효소는 회복되지 않았다. 각 수은제들이 억제물질로 작용할 때 K_i값과 억제양상이 측정되었는데 모두 경쟁적 방해를 보였다. K_i값은 10 mM 인산완충용액에서 측정한 것이 100 mM 인산완충용액에서 측정한 것보다 훨씬 낮아 인산기가 기질이 아니어도 효소의 부착에 큰 영향을 미치는 것을 보여주고 있다.