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Purification and Some Properties of Arginine Deiminase in *Euglena gracilis* Z

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Euglena gracilis Z로부터 Arginine Deiminase의 정제 및 그의 특성

초 록

Euglena gracilis 에서 arginine deiminase는 mitochondrial matrix 내에 존재한다. 고도로 정제된 효소가 0.23 mM의 K_m 값을 갖고 효소반응을 하기 위해서는 Co^{2+}가 필요하며, 이때 최적 pH는 9.3~10.3이었다. Gel filtration에 의해서 얻어진 조효소 단백질의 분자량은 87,000이었으며, SDS-acrylamide gel electrophoresis에 의해 효소는 48,000의 분자량을 갖는 2개의 동일한 subunit로 구성되어 있음이 밝혀졌다. *Euglena*의 arginine deiminase는 sulfhydryl inhibitors에 의해서 활성이 저지되었는데, 이는 sulfhydryl group이 효소의 활성부위에 관여함을 나타낸다. 이 sulfhydryl group은 arginine이 효소와 결합하는데 있어서 negative cooperativity를 나타내었다. β-guanidinopropionate, γ-guanidinobutyrate와 guanidinosuccinate는 효소의 활성을 저지시키지 않는데 반하여, L-α-amino-β-guanidino-propionate, D-arginine, 그리고 L-homoarginine은 효소의 활성을 강력하게 저지시켰다. Citrulline과 ornithine에 의해서도 상당한 정도의 효소활성저지가 관찰되었다. 우리는 *Euglena*의 arginine deiminase의 독특한 성질이 *Euglena* 라는 원생동물 내에서 arginine 대사의 조절에 어떻게 영향을 미치는지를 토의하고자 한다.

ABSTRACT

In *Euglena gracilis* arginine deiminase was located in the mitochondrial matrix. The highly purified enzyme required Co^{2+} for the enzyme reaction with the K_m value of 0.23 mM, and its optimum pH was 9.7 to 10.3. The molecular weight of the native enzyme protein was 87,000 by gel filtration, and SDS-acrylamide gel electrophoresis showed that the enzyme consisted of two identical subunits with a molecular weight of 48,000. *Euglena* arginine deiminase was inhibited by sulfhydryl inhibitors, indicating that a

sulfhydryl group is involved in the active center of the enzyme. It exhibited negative cooperativity in binding with arginine. L-α-amino-β-guanidino-propionate, D-arginine, and L-homoarginine strongly inhibited the enzyme while β-guanidinopro-pionate, γ-guanidinobutyrate, and guanidinosuccinate did not. Considerable inhibition was also observed with citrulline and ornithine. We discuss the effects of the unique properties of the *Euglena* arginine deiminase on the regulation of arginine metabolism in this protozoon.

Key words—*Euglena gracilis*, Arginine deiminase, Gel electrophoresis, Inhibition, Arginine metabolism.

INTRODUCTION

Euglena gracilis accumulates arginine as the major nitrogen reserve depending on the phase of growth when a relatively excess of a nitrogen source is supplied, and upon acquiring a carbon source the amino acid is rapidly metabolized for renewed cell growth (Park *et al.*, 1983). Arginine as a cellular nitrogen reserve has been reported in higher plants (Etten *et al.*, 1967) and *Neurospora* (Weiss, 1976). In *Euglena* arginine is catabolized to ornithine via citrulline by the action of arginine deiminase and citrullinase, or by the so-called arginine dihydrolase pathway (Park *et al.*, 1983).

Many reports have dealt with the occurrence of arginine deiminase in prokaryotes (Weiss *et al.*, 1977; Petrack *et al.*, 1957; Shibatani *et al.*, 1975) and eukaryotes (Roche *et al.*, 1967;Hill *et al.*, 1967; Sussenbach *et al.*, 1969) and there are a few reports on the purification of this enzyme in bacteria (Petrack *et al.*, 1957; Shibatani *et al.*, 1975) but no information is available on the purification and detailed properties of the enzyme in eukaryotic organisms. In the present paper the purification and some properties of arginine deiminase in *E. gracilis* are described and the significance of the enzyme in the regulation of arginine metabolism in *Euglena* is discussed.

MATERIALS AND METHODS

Organism and culture. E. gracilis strain Z was grown under illumination (2500 lux) at 27℃ as described previously (Park et al., 1983).

Fractionation of cell homogenate. A cell homogenate was obtained by partial trypsin digestion of the pellicle followed by mild mechanical disruption (Todunaga *et al.*, 1979), and fractionated by differential centrifugation according to Shigeoka *et al.* (Shigeoka *et al.*, 1979)

Preparation of crude enzyme. A culture of *E. gracilis* in the lateexponential phase of growth (10^7 cells/ml) was supplemented with 200 mM ethanol (final concentration), and after 12 hr of incubation it was centrifuged at 2000×g for 5 min to harvest cells. The cells (24 g) were washed twice with distilled water, suspended in 10 ml of 10 mM potassium phosphate buffer, pH 7.0, and disrupted by sonication (10kc) for a total of 3 min with 11 intervals of 15 sec each. The supernatant obtained by centrifugation of the sonicate at 9000×g for 15 min was used as a crude enzyme. The typical preparation showed an enzyme activity of 0.38 μmol of citrulline formed per mg protein per min, when the arginine concentration was 50 mM.

Protein was determined by the method of Bradford (Bradford *et al.*, 1976).

Enzyme assays. Deiminase as assayed by measuring labeled citrulline formed from [U−^{14}C] arginine (0.1 μCi/mM) by the enzyme reaction as described previously (Park *et al.*, 1983). Succinate semialdehyde dehydrogenase, a mitochondrial marker enzyme (Tokunaga *et al.*, 1976), glucose 6-phosphatase, a microsomal marker enzyme (Oda *et al.*, 1981); ribulose-1,5-bisphosphate carboxylase, a chloroplast-marker enzyme (Oda *et al.*, 1981); and glutamate dehydrogenase, a cytosolic marker enzyme (Todunaga *et al.*, 1979) were assayed by the respectively cited methods.

Molecular weight determination by gel filtration. The molecular weight of the enzyme was determined by the method of Andrews (Andrews *et al.*, 1964) using a column (1.5×60 cm) of Sephacryl S-200. Chromatography was carried out at 4℃ at a flow rate of 30 ml per hr using 100 mM potassium phosphate buffer, pH 7.4, as an eluant. The column was calibrated with lactate dehydrogenase (pig heart), malate dehydrogenase (pig heart), trypsin inhibitor (soybean), and cytochrome c (horse heart).

SDS-Polyacrylamide gel electrophoresis. SDS-Polyacrylamide gel electrophoresis of the purified arginine deiminase was performed according to Weber and Osborn (Weber *et al.*, 1969). The enzyme and known protein standards (Electrophoresis calibration kit, Pharmacia Fine Chemicals) were treated by heating for 5 min at 100℃ in a solvent system consisting of 1% SDS, 1% 2-mercaptoethanol and 10 mM sodium phosphate buffer, pH 7.2. Electrophoresis was carried out at a constant current (5 mA/gel) with bromophenol blue as a migration marker. Proteins in the gel were stained with Coomassie brilliant blue R-250 and destained in 7% acetic acid.

RESULTS

Subcellular location of arginine deiminase

Distribution of arginine deiminase and

marker enzymes in the subcellular fractions of *E. gracilis* is shown in Table 1. The deiminase was located in the mitochondrial fraction, as judged from the distribution of activities of marker enzymes. Sonication of the isolated mitochondrial fraction released the deiminase as well as succinate semialdehyde dehydrogenase into the 100,000 ×g supernatant. The slight amount of the deiminase in the chloroplast fraction was due to contamination by mitochondria.

Purification of arginine deiminase

Typical purification steps of the *Euglena* arginine deiminase are summarized in Table 2. The crude enzyme was centrifuged at 105,000×g for 60 min with a Hitachi 65P ultracentrifuge, and the supernatant was applied onto a DEAE-cellulose column (3×20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After washing with 50 ml of the same buffer the column was eluted with a linear concentration gradient (0~0.3 M) of potassium chloride and active fraction

Table 1. Distribution of arginine deiminase and marker enzymes in subcellular fractions of *E. gracilis*

	Enzyme activity[a]			
	Cytosol	Mitochondria	Microsomes	Chloroplasts
Arginine deiminase	7.4 (0.03)[b]	78.0 (1.53)[b]	3.9 (0.13)[b]	13.6 (0.51)[b]
Glutamate dehydrogenase ($NADP^+$)	94.1	0	0	0
Succinate semialdehyde dehydrogenase ($NADP^+$)	5.1	86.9	0	3.5
Glucose 6-phosphatase	0	0	27.3	0
Ribulose-1,5-bisphosphate carboxylase	7.5	9.9	3.7	76.0

a % against the activity in the crude extract.

b Specific activity, μ mol/mg protein/min.

Table 2. Purification steps of *Euglena* arginine deiminase.

	Total protein (mg)	Specific activity (μ mol/mg protein/min)	Total activity (μ mol/min)	Recovery (%)
Crude extract	1202	0.0284	14.14	100
Ultracentrifugation	584.0	0.0345	20.15	59
DEAE-Cellulose	35.75	0.466	16.66	49
P-Cellulose	11.38	1.38	15.74	46
Sephacryl S-200	5.04	1.59	8.03	24

were brought to 70% saturation of ammonium sulfate. The precipitate was dissolved in the above buffer and passed through a Sephadex G-25 column (2.5× 45 cm) to remove the ammonium sulfate. The protein fraction was applied onto a column (2×15 cm) of P-cellulose equilibrated with 10 mM potassium phosphate buffer, pH 7.4, and the column was eluted with a 10 to 200 mM gradient of potassium phosphate buffer, pH 7.0. The unadsorbed, active fractions were precipitated by the addition of ammonium sulfate to 70% saturation, and the precipitate was dissolved in 50 mM phosphate buffer, pH 7.0. The enzyme solution was applied onto a Sephacryl S-200 column (2.5×55 cm) and eluted with the 50 mM buffer. The elution pattern (Fig. 1) showed only one peak of deiminase activity, and the

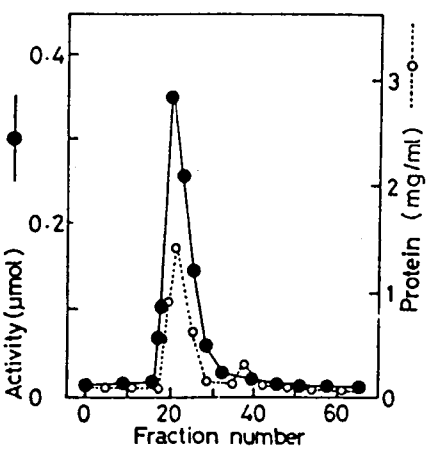

Fig. 1. Elution pattern by sephacryl S-200 gel filtration.

active fractions (Nos. 19 through 27) were combined and stored at -20℃. The enzyme preparation thus obtained had been purified 56-fold over the crude enzyme, giving 24% overall recovery of the activity. The preparation exhibited only one detectable protein band in SDS-polyacrylamide disc gel elec-trophoresis.

Determination of molecular weight

Figure 2 shows plots of the elution volume from a Sephacryl S-200 column against logarithms of the molecular weight of the standard proteins and arginine deiminase. The molecular weight of this enzyme was calculated to be about 87,000. SDS-polyacrylamide gel electrophoresis of the deiminase together with several proteins with known molecular weight indicated that the subunit of this enzyme had a molecular weight of 48,000, as shown in Fig. 3.

Effects of temperature and pH

This enzyme was stable up 40℃ after treatment for 15 min, and retained 61.2% of its activity after treatment at 50℃ (Fig. 4).

Figures 4 and 5 show that the maximum activity of the enzyme is obtained at 30℃ and pH 9.7~10.3. The enzyme was stable in the range of pH 8.0 to 11.0 when treated at various pHs for 15 min at 40℃ before the enzyme

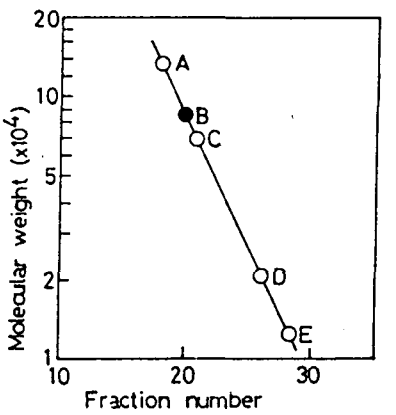

Fig. 2. Molecular weight determination by gel filtration. A Sephacryl S-200 column was calibrated with known protein standards, including lactate dehydrogenase (135,000) (A), malate dehydrogenase (70,000) (C), Trypsin inhibitor (20,100) (D), and cytochrome c (12, 400) (E) (B), Euglena arginine deiminase.

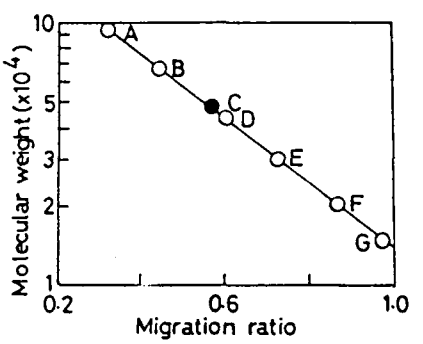

Fig. 3. Molecular weight estimation by SDScacrylamide gel electrophoresis. A, phosphorylase b (94,000); B, bovine serum albumin (67,000); C, Euglena arginine deiminase; D, ovalbumin (43,000); E, carbonic anhydrase (30,000); F, soybean trypsin inhibitor (20,100); G, α-lactalbumin (14,400).

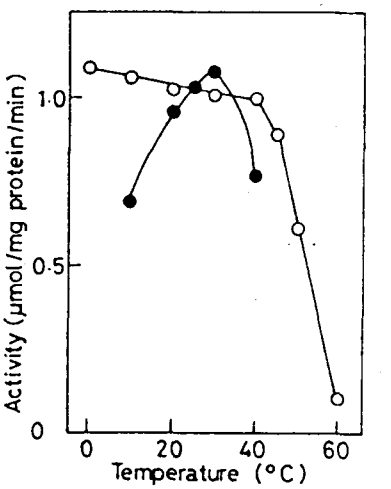

Fig. 4. Effect of temperature on arginine deiminase. For determining temperature optimum (●) enzyme was assayed under standard conditions but at various temperatures, and for temperature stability (○) enzyme was kept for 15 min at varied temperatures before assaying.

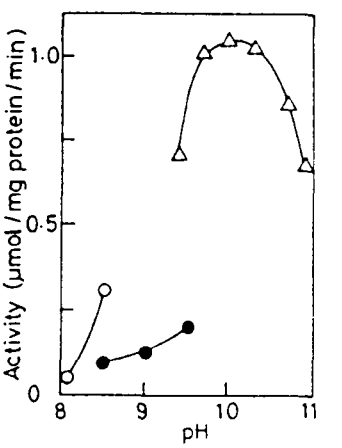

Fig. 5. pH optimum of arginine deiminase. Enzyme was assayed in 50 mM each of potassium phosphate buffer (○), glycine-KOH buffer (●) or sodium bicarbonate buffer (△) under standard assay conditions but at different pHs.

for 15 min at 40℃ before the enzyme reaction.

Effects of metal ions and some compounds

As shown in Table 3 Co^{2+} was essential to the enzyme activity, and Mg^{2+} could replace Co^{2+} with lower activity. Other metal ions tested hardly affected the enzyme activity. The activation of the enzyme by Co^{2+} was completely removed by EDTA. Figure 6 shows that 0.5mM $CoCl_2$ gives the maximum enzyme activity, and that the K_m value is 0.23 mM.

Table 3. Requirement of Metal Ions for Enzyme Activity

Addition (1 mM)	Enzyme activity (μ mol/mg protein/min)
None	0.12
$CoCl_2$	1.83
$MgCl_2$	0.78
$MnCl_2$	0.12
$MaCl_2$	0.15
$NiCl_2$	0.14
$FeSO_4$	0.14
$CoCl_2$+EDTA	0.15

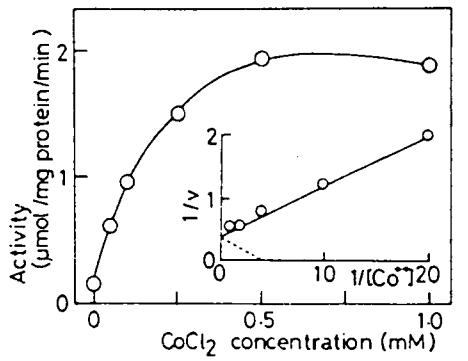

Fig. 6. Effect of $CoCl_2$ concentration on arginine deiminase.

Table 4. Effects of sulfhydryl reagents and vitamin B_6-enzyme inhibitors

Addition[a]	Enzyme activity (μ mol/mg protein/min)	Inhibition (%)
None	1.19	0
p-Chloromecuribenzoate (0.1 mM)	0.11	90.8
5,5′-Dithiobis (2-nitrobenzoate)	0.28	86.5
N-Ethylmaleimide	0.83	30.3
$HgCl_2$	0.33	72.3
Mersaryl	0.22	81.5
Mersaryl+2-merca -ptoethanol (2 mM)	1.36	-14.3
Isonicotinic acid hydrazide	1.25	-5.0
Aminoxyacetate	1.14	4.2
Semicarbazide	1.18	0.8

a At 1 mM unless otherwise specified.

Euglena arginine deiminase was inhibited 77~82% by sulfhydryl inhibitors like pchloromercuribenzoate, 5,5′-dithiobis-(2-nitrobenzoate), N-ethylmaleimide, Hg^{2+}, and mersaryl, and the enzyme was protected from this inhibition by 2-mercaptoethanol, indicating that a sulfhydryl group in the enzyme protein is concerned with the active center of the enzyme. Isonicotinic acid hydrazide, aminoxyacetic acid, and semicarbazide, the inhibitors of vitamin

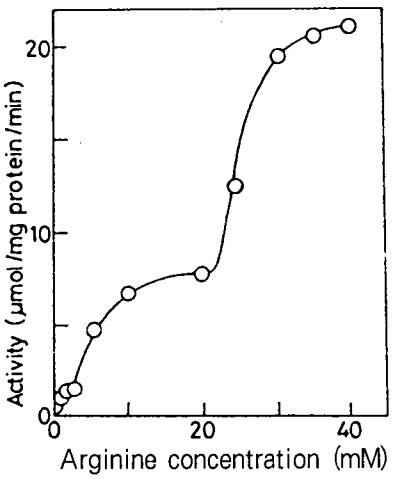

Fig. 7. Effect of Arginine Concentration on Enzyme Activity.

Table 5. Effects of arginine analogues and related amino acids

Addition (1 mM)	Enzyme activity (μ mol/mg protein/min)	Inhibition (%)
None	1.10	0
Guanidine HCl	1.03	6.8
Guanidinoacetate	1.05	5.0
β -Guanidinopropionate	1.10	0
γ -Guanidinobutyrate	0.88	20.1
Guanidinosuccinate	1.13	-3.0
L-α -Amino-β -guanidino-propionate	0.29	74.0
D-Arginine	0.42	61.5
L-Homoarginine	0.48	56.8
Agmatine	0.98	11.1
Arginosuccinate	0.96	12.7
Citrulline	0.51	53.5
Homocitrulline	0.64	42.1
Ornithine	0.33	70.4
Apartate	1.12	-2.0
Glutamate	0.87	20.6
Proline	0.74	32.4
Fumarate	1.14	-4.0
Urea	0.87	21.2

B6 enzymes, hardly affected the deiminase activity (Table 4). The *Euglena* enzyme was distinct from the *Streptococcus* enzyme 5) in that it was not inhibited by carbonyl reagents.

Effects of substrate concentration and arginine analogues

Figure 7 shows that the activity of arginine deiminase exhibits triphasic proportion when the arginine concentration was varied, indicating that the enzyme has plural combining sites with different affinities for arginine.

Effects of some L-arginine analogues containing guanidino groups and nitrogenous compounds related to arginine metabolism on the enzymatic hydrolysis of arginine are shown in Table V. L-α-amino-β-guanidinopropionate, D-arginine, and L-homoarginine strongly inhibited the hydrolysis while β-guanidinopropionate, γ-guanidinobu-tyrate and guanidino-succinate, all containing no α-amino group, hardly affected the reaction at 1 mM. Cit-rulline, homocitrulline and ornithine strongly inhibited the enzyme reaction while proline, glutamate and urea had slightly inhibitory effects.

DISCUSSION

Arginine deiminase in *E. gracilis* was found to be located in mitochondria from

its parallel distribution with succinate semialdehyde dehydrogenase, a marker enzyme for the mitochondrial matrix. Parallel behavior of the two enzymes in sonication of isolated *Euglena* mitochondria supports the conclusion. Hill and Chambers (Hill *et al.*, 1967) have reported that the deiminase in *Tetrahymena* is located in the 100,000×g supernatant of freeze-thawed cells.

Arginine deiminase of *E. gracilis* was purified about 56-fold. This is the first case of purification of the deiminase to homogeneity from an eukaryote. Properties of this enzyme have been studied mainly in bacteria, such as *Mycoplasma* (Weickmann, 1977) and *Pseudomonas* (Shibatoni *et al.*, 1975). The molecular weight of the purified *Euglena* enzyme was estimated to be 87,000 by gel filtration, and the result of acrylamide gel electrophoresis in the presence of SDS suggested that the enzyme consisted of two apparently identical subunits with a molecular weight of 48,000. The enzyme of *Mycoplasma hominis* (Weickmann *et al.*, 1977) has a molecular weight of 78,300 and that of *Pseudomonas putida* (Shibatani *et al.*, 1975) 120,000. The *Psedomonas enzyme* was shown to be consisted of two identical subunits (Shibatani *et al.*, 1975).

Euglena deiminase showed its highest activity at pH 9.7 to 10.3, distinct from the enzymes from yeast (Roche *et al.*, 1967), bacteria (Shibatani *et al.*, 1975), *Chlorella 1* (Hill *et al.*, 1967) and Mycoplasma (Weichmann *et al.*, 1977) which have optimum pHs at 6.0 to 6.7. The *Tetrahymena* enzyme (Hill *et al.*, 1967) is most active at pH 9.3 and *Chlamydomonas* enzyme (Sussenbach *et al.*, 1969) at 8.0~8.6. the *Euglena* deiminase required Co^{2+} for the reaction and the K_m value for Co^{2+} was 0.23 mM. The metal ion requirement of arginine deiminase is reported here for the first time. Roche and Lacombe (Roche *et al.*, 1967) reported that arginine deiminase obtained by plasmolysis of baker's yeast was inactivated by dialysis and reactivated by the addition of Co^{2+} or Ni^{2+}, but that such an effect of metalions was not found in the alcohol-fractionated enzyme from acetone-treated yeast. They concluded that the effect was due to stabilization of the active structure of the enzyme by the metal ion.

Euglena ardinine deiminase was markedly inhibited by sulfhydryl reagents, and the inhibition was removed by 2-mercaptoethanol, indicating that a sulfhydryl group is involved in the active site of the enzyme. The *Pseudomonas* enzyme (Shibatani *et al.*, 1975) has also been reported to be inhibited by sulfhydryl inhibitors.

Euglena deiminase was revealed to be unique in that negative cooperativity

was observed, indicating that substrate-induced conformational changes are responsible for the regulation of the activity of this enzyme (Levitzki *et al.*, 1969) In a typical experiment, when *Euglena* cells reached the early exponential phase of growth the intracellular concentration of arginine was 190 mM (Park *et al.*, 1983), and the amino acid decreased markedly in the mid-exponential phase; in the late exponential phase the arginine level increased again rapidly to reach about 30 mM. When ethanol was added as a carbon source in the late exponential phase, the size of the arginine pool was rapidly decreased and renewed cell division took place. These facts indicate that arginine is accumulated as a nitrogen reserve in *Euglena*, and its rapid accumulation and mobilization along with cell growth is apparently regulated by a unique mechanism depending on the characteristic property of arginine deiminase. It has been found that phosphoenolpyruvate carboxylase in *Escherichia coli* (Corwin *et al.*, 1968) and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Conway *et al.*, 1968) exhibit negative cooperativity in the binding with substrate. Effects of L-arginine analogues containing guanidino groups are classified into the two following groups: (a) L-α-amino-β-guanidinopropionate, D-arginine, and L-homoarginine, which are α-amino acids and strongly inhibit arginine deiminase activity and (b) β-guanidinopropionate, γ-guanidinobutyrate, and granidinosuccinate which contain no α-amino group and hardly inhibit the enzyme. The results suggest that an α-amino group is essential for the binding of arginine or an analogue to the catalytic site of the enzyme. Shibatani et al. (Shibatani *et al.*, 1975) have reported that the arginine deiminase from *P. putida* is not inhibited by D-arginine. Inhibition of *Euglena* arginine deminase by citrulline and ornithine indicates that these compounds serve as regulatory end products in the arginine dihydrolase pathway.

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