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Affinity labeling of the Vacuolar Arginine Transporter in *Neurospora crassa*

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*Neurospora crassa*의 액포에 존재하는 arginine transporter의 표지방법

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ABSTRACT: Based on the specificty of recognition of the vacuolar arginine transporter, N-*p*-nitrobenzoxycarbonyl (NBZ)-L-arginyl diazomethane was synthesized and used as an affinity label specific for the arginine transporter. This arginyl derivative inhibited both ATP-dependent and independent L-arginine transport into vacuolar membrane vesicles. When vacuolar proteins were labeled with radioactive NBZ arginyl diazomethane, the binding was irreversible, detached by treatment with base and blocked by treatment with cysteinyl blocking groups suggesting cysteine as a labeling site.

KEY WORDS □ Vacuole, NBZ-arginyl diazomethane, arginine transporter.

In most prokaryotes, the pool size of many endogenous amino acids are often maintained at low levels, and catabolic enzymes remain uninduced. In eukaryotic cells, however, endogenous amino acids frequently accumulate despite the presence of significant levels of catabolic enzymes. These large pools are often localized in vacuoles and are not catabolized. (Matile, 1978; Huber-Wälchi and Weimken, 1979).

Vacuoles are the largest membrane bound structures of plant and fungal cells occupying 25-95% of the cell volume. Vacuoles maintain cytoplasmic homeostasis of nutrients and act as metabolic or lytic compartments in intracellular digestive processes in addition to their mechanical functions of maintaining turgor pressure and mechanical support by accumulating various kinds of solutes and ions (Matile, 1978; MacRobbie, 1979).

One of the most important roles of vacuoles is their storage function. In *Neurospora crassa* and *Saccharomyces cerevisiae,* vacuoles contain several proteases, ribonucleases, alkaline phosphatases, α-mannosidase, and the bulk of basic amino acids (Matile, 1978; Vaughn and Davis, 1981). While most of the arginine (98%) of *N. crassa* is kept in the vacuoles (Weiss, 1976), the small cytosolic arginine pool can expand and contract quickly in response to changes in the availability of exogenous arginine by preventing the free exchange of amino acids between the vacuolar and cytoplasmic compartments. Thus movement of arginine across the vacuolar membrane can influence arginine metabolism by separating the enzymes of arginine catabolism from the main pool of arginine and by serving as a reserve pool of arginine to be used in case of nitrogen starvation (Huber-Wälchi and Wiemken, 1979; Legerton and Weiss, 1984). Thus arginine compartmentation in vacuoles function as a buffer to maintain the cytoplasmic arginine concentration relatively constant under different gro-

wth conditions. In *N. crassa, in vivo* experiments have shown that metabolic energy is required for the transport of arginine into the vacuoles, but not for its retention (Drainas and Weiss, 1982a and 1982b). *In vitro* experiments showed ATP-dependent and independent arginine uptake mechanism with narrower specificity than those in *S. cerevisiae* (Paek and Weiss, 1989). The specificity of L-arginine transport in *S. cerevisiae* is quite broad and accomplished at least by three different mechanisms. Mobilization of L-arginine through vacuolar membrane is also inhibited by inhibitors or an uncoupler of respirations, but not by an inhibitor of glycolysis. Thus mobilization of arginine from vacuoles also requires metabolic energy. Several receptor proteins have been identified and isolated using separation by detegent and reinsertion into a lipid bilayer (Oomen *et al.*, 1987). However, L-arginine would most likely be detached from the arginine transporter in the presence of detergent and the arginine binding activity of the arginine transporter might be lost after solubilization from membrane. Thus an alternative approach was conceived to identify and purify the arginine transporter. Since an intact guanido side chain and L-configuration are important for the specificity of recognition by the arginine transporter, NBZ arginyl diazomethane was synthesized and used to indentify the arginine transporter (Paek and Weiss, 1989). In this paper, its binding mechanism to the arginine transporter was studied to find out the labeling site and how labeling was accomplished.

MATERIALS AND METHODS

Growth conditions.

The wild-type *Neurospora crassa* strain LA1 (74A, FGSC 987) was used in this study. Mycelia were grown in Vogel's medium (Vogel, 1964) containing 1.5% sucrose in 12*l* carboys with vigorous aeration and stirring at 30°C. Conidia were inoculated to give 10^6 conidia/ m*l* culture medium.

Vacuole isolation and preparation of vacuolar membrane vesicles.

Vacuoles and vacuolar membrane vesicles were prepared following the procedures described in a previous paper (Paek and Weiss, 1989).

Arginine transport assay.

Transport assay buffer (25 mM Tris/MES, 1.2 mM $MgCl_2$, 0.3 mM ATP, pH 7.8) was prepared as a 5 x concentrated solution and divided into small aliquots and kept frozen at -70°C until use. Just before assay, 0.02 m*l* of transport assay buffer was mixed with 0.01 m*l* of 3 mM L-[2,3-^{3}H] arginine (100 mCi/mmol). Water was added up to 0.08 m*l* for each assay tube. The prepared assay mixture was warmed up to 30°C before adding vacuolar membrane vesicles or vacuoles. Vacuolar membrane vesicles (1-10 μg in 0.02 m*l*) or vacuoles (20 μg in 0.02 m*l*) were added and incubated at 4°C or 30°C for the designated time. After incubation, transport was stopped by dilution with 5 m*l* of ice cold buffer C (25 mM MES/Tris, 1.2 mM $MgCl_2$, pH 6.9). The reaction mixture was then filtered through membrane filter (Millipore type HA, 0.45 μm) and washed with an additional 10 m*l* of ice cold buffer C using a Vacuum manifold (Millipore). Before filtration, membrane filters were wetted with 10% methanol and kept in 1 M L-arginine until use. After filtration, filters were dried under a heat lamp for 5 min. Radioactivity remaining on the filters was determined using 10 m*l* of Liquiscint scintillation fluid (National Diagnostics) in a Beckman LS8100 liquid scintillation spectrometer.

Protein determination.

The amount of protein was determined using a BCA kit (Pierce) using bovine serum albumin as the standard. Fifty μ*l* of sample was mixed with 1 m*l* of assay mixture and incubated for 2 hours at room temperature or until color developed at 50°C. The absorbance was mearsured at 562 nm.

Synthesis of NBZ arginine.

The procedure for the synthesis of NBZ arginyl diazomethane is described in a previous paper (Paek and Weiss, 1989).

Synthesis of radioactive NBZ arginyl derivatives.

L-[2,3-^{3}H] argininie monohydrochloride (50 Ci/mmol) dissolved in 0.1 N HCl was added to 10 m*l* of 1 N sodium bicarbonate and the pH was

adjusted to 10.0 with 4 N NaOH. *p*-Nitrobenzyl chloroformate (0.05g) was dissolved in 3 m*l* of dioxane and the solution was added to the arginine solution at room temperature. To keep the pH above 9, 4 N NaOH was added intermittently to the reaction mixture. After 1 hour of stirring, the pH of the mixture was slowly adjusted to pH 5.5 with concentrated hydrochloric acid. The small amount of the disubstituted product was removed by filtration through a fine fritted glass funnel. The filtrate was extracted 3 times with 10m*l* of ethyl acetate. The pH of the aqueous phase was then adjusted to 7.5 with a few drops of 4 N NaOH. The clear solution was dried completely under reduced pressure (rotary evaporator, 30°C, 0.3 torr). The precipitate was washed with cold water and cold 95% ethanol. The product was dissolved in a minimum amount of warm water in a 50 m*l* round bottom flask and dried completely in a lyophilizer.

Synthesis of radioactive NBZ arginyl chloride.

All reactions in the following steps were carried out under N_2 atmosphere. NBZ arginyl monohydrochloride (1 μmol) in the same flask used for lyophilization was overlayed with 5 m*l* of cold thionyl chloride. The mixture was vigorously stirred at room temperature for 5 min. The crystalline starting material was rapidly converted to a heavy oil. The flask was placed in an ice-salt bath (-18°C) and the stirring was continued for about 10 min. The excess thionyl chloride was removed and the oily product was stirred with 30 m*l* of cold anhydrous ether which caused it to solidify. The white solid product was washed several times with cold anhydrous ether and than dried briefly under reduced presure.

Synthesis of radioactive NBZ arginyl diazomethane.

Radioactive NBZ arginyl chloride hydrochloride was dissolved in anhydrous tetrahydrofuran (15 m*l*) at 0°C and an ethereal solution of diazomethane was added until yellow color persisted. The mixture was stirred for 1 hour in an ice bath under N_2 and then 30 m*l* of cold anhydrous ether was added. The light yellow precipitate was washed several times with cold anhydrous ether and dried briefly under reduced pressure. The final product was kept at -20°C under N_2 in the absence of light.

Synthesis of diazomethane.

Diazomethane was obtained by the previously published method (Arndt, 1943).

Labeling vacuolar proteins with NBZ arginyl diazomethane.

Reaction mixtures containing vacuoles or vacuolar membrane vesicles in arginine transport assay buffer were incubated at 30°C or irradiated with UV in the presence of NBZ arginyl diazomethane or radioactive one. For UV irradiation, reaction mixtures were transferred to pyrex tubes to absorb the low wavelength UV. The tubes were attached to a cooling jacket and irradiated with a UV lamp (Hanovia) for 15 min in a cold room (4°C).

Measurement of binding of NBZ arginyl diazomethane.

Vacuolar membrane vesicles in arginine transport assay buffer were labeled with radioactive NBZ arginyl diazomethane by UV irradiation or incubation at 30°C as described above. After the designated time, the vesicles were collected by centrifugation at 100,000 × g (24,000 rpm) for 30 min in a SW 27 rotor. The vesicles were then washed with the same buffer by dispersion and centrifugation. The washed vesicles were dissolved in 1 m*l* of 5% sodium dodecyl sulfate in a boiling water bath. The totally solubilized samples were transferred into scintillation vials and incubated at 50°C for 3 hours with 10 m*l* of Fluorsol scintillation fluid (National Diagnostics). The vials were then cooled to room temperature and radioactivities were measured in a Beckman LS8100 liquid scintillation spectrometer.

Acid precipitation of proteins.

Trichloroacetic acid was added to the labeling reaction mixtures to a final concentration of 10%. Contents were mixed vigorously and kept for 1 hour in an ice bath and filtered through glass fiber filters (Whatman, GF/H). The filters were washed with 1 m*l* of each of 5% trichloroacetic acid, ethanol/ethyl ether(1:1, v v), and ethyl ether, and then dried in air. Filters were placed in scintilation vials and incubated for 3 hours at 50°C in 10 m*l* of Fluorosol scintillation fluid. The vials were

cooled to room temperature and then radioactivity was mesured in a liquid scintillation spectrometer.

Acetone precipitation of proteins.

After vacuolar proteins were labeled as described above, 8 volumes of cold acetone (-20°C) was added to the reaction mixture. The mixture was stirred briefly and incubated for 30 min in an ice bath. It was then centrifuged for 30 min at high speed in a desk-top clinical centrifuge at -20°C. The protein precipitate was washed with 1 m*l* of cold acetone (-20°C) and flushed with N_2 gas to remove acetone. To each pellet, 1 m*l* of 5% sodium dodecyl sulfate was added and the mixture was boiled for 10 min in a boiling water bath. Radioactivity was measured using 10 m*l* of Liquiscint scintillation fluid as described above.

Base treatment of acid precipitated labeled vacuolar proteins.

Vacuolar membrane vesicles (10 μg) were labeled with radioactive NBZ arginine diazomethane (0.4 μM, 50 Ci/mmol) in 0.4 m*l* of arginine transport assay buffer by incubation at 30°C for 10 min. Vacuolar proteins were then treated with 0.1 m*l* of 2 M NH_2OH or 1 M NaOH. After 10 min incubation at room temperature, proteins were precipitated with 0.5 m*l* of concentrated trichloroacetic acid. Samples were mixied vigorously and incubated for 1 hour in an ice bath, filtered through Whatman filter papers (GF/H) and washed with 1 m*l* each of 5% trichloroacetic acid, ethanol/ethyl ether (1:1, v/v), and ethyl ether. After drying briefly in air, filters were placed in 5 m*l* of Fluorosol scintillation fluid (National Diagnostics), incubated 3 hour at 50°C, and cooled to room temperature. Radioactivity was measured in a liquid scintillation spectrometer.

Treatment with cysteinyl blocking reagents.

Vacuoles (1 mg) were incubated in arginine transport assay buffer containing 10 mM iodoacetic acid or 10 mM NEM (N-ethylmaleimide) for 10 min at 30°C. NBZ arginyl diazomethane (0.4 μM, 50 Ci/mmol) was added to the reaction mixtures, and the reaction mixtures were irradiated with UV light as described above. After 15 min of irradiation, trichloroacetic acid was added to a fianl concentration of 10%, and the resulting suspension was mixed vigorously and incubated for 1 hour in an ice bath. Radioactivity associated with the protein precipitate after filtration was measured as described above.

RESULTS

Arginine transport in the presence of NBZ arginyl diazomethane.

Since NBZ arginyl diazomethane had an intact guanido side chain and L-configuration, it was expected to be recognized by the arginine transporter and inhibit arginine transport. When vacuolar membrane vesicles were incubated at 4°C or 30°C in the presence of NBZ arginyl diazomethane, arginine transport was strongly inhibited at both temperatures. Arginine transport at 30°C in the presence of 5 mM NBZ arginyl diazomethane was only 14% of the transport in the absence of this derivative and no transport was observed at 20 mM NBZ arginyl dizomethane (Table 1) compared 17% at 20 mM L-arginine (Paek and Weiss, 1989). This result suggested that NBZ arginyl diazomethane might irreversibly bind to the arginine transporter preventing reversible binding of L-arginine. To test this assumption, vacuoles were preincubated with NBZ arginyl diazomethane before radioactive L-arginine was added to the reac-

Table 1. *Arginine transport in the presence of NBZ arginyl diazomethane.*

Vacuolar membrane vesicles (10 μg) were incubated with 0.3 mM radioactive L-arginine (100 mCi/mmol) in arginine transport assay buffer in the presence of different concentrations of NBZ arginyl diazomethane. After 10 min at 30°C or 60 min at 4°C, the reactions were stopped and the amount of transported L-arginine was measured as described in Materials and Methods.

		Transported Arginine		
		NBZ Arginyl Diazomethane		
Temperature (°C)	Incubation (min)	0 mM	5 mM	20 mM
		(cpm)[a]		
30	10	2307	325	0
4	60	1717	862	-[b]

[a] Background (0 min incubation) was subtracted.
[b] Not measured.

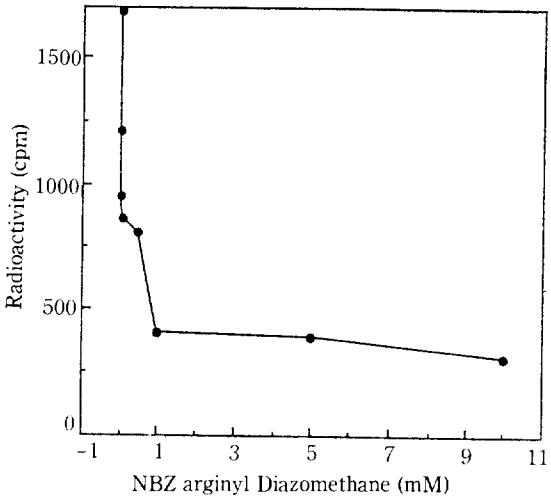

Fig. 1. *Arginine transport after treatment of vacuoles with different concentrations of NBZ arginyl diazomethane*

Vacuoles (20 μg) were incubated at 30 °C for 10 min with various concentrations of NBZ arginyl diazomethane in arginine transport assay buffer. L-Arginine (0.3 mM, 100 mCi/mol) was added to the reaction mixtures and incubation was continued an additional 10 min. Transported arginine was measured as described in Materials and Methods. [Background (0 min incubation, about 350 cpm) was not substracted.]

tion mixture. Arginine transport after preincubation was completely inhibited even at 1 mM NBZ arginyl diazomethane (Fig. 1). Better inhibition with preincubation suggested irreversible binding of NBZ arginyl diazomethane to the arginine transporter.

Binding of NBZ arginyl diazomethane to vacuolar proteins.

When vacuolar proteins were incubated at 30°C or irradiated with UV in the presence of radioactive NBZ arginyl diazomethane, radioactivity derived from NBZ arginyl diazomethane is clearly associated with vacuolar membrane vesicles (Table 2). Radioactivity was not removed from the vesicle by treatment with acid or acetone (Table 3). Since acid or acetone treatment should remove any ionic binding between arginine derivatives and vacuolar proteins, the radioactivity remaining with the protein precipitate must be due to covalent binding.

Table 2. *Binding of NBZ arginyl diazomethane to vacuolar membrane vesicles.*

Vacuolar membrane vesicles (10 μg) in arginine transport assay buffer were labelled with radioactive NBZ arginyl diazomethane (0.4 μM, 50 Ci/mmol) by irradiation with UV or incubation at 30 °C. After 10 min, reaction mixtures were centrifuged and washed with arginine transport assay buffer. Radioactivity associated with vesicle pellets was measured as described in Materials and Methods.

	Radioactivity associated with vacuolar membrane vesicles
	(cpm)
No Treatment	350
UV Irradiation	811
Incubation at 30 °C	884

Table 3. *Covalent binding of NBZ arginyl diazomethane to vacuolar proteins.*

Vacuolar proteins (1 mg) were labelled with radioactive NBZ arginyl diazomethane (0.4 μM, 50 Ci/mmol) in arginine transport assay buffer by UV irradiation as described in Materials and Methods. Proteins were precipitated by addition of trichloroacetic acid or acetone as described in Materials and Methods. Radioactivity associated with protein precipitates was measured as described in Materials and Methods.

Treatment	Radioactivity associated with protein precipitate
	(cpm)
Trichloroacetic Acid	34000
Acetone	44580

Base treatment of vacuolar proteins labeled with radioactive NBZ arginyl diazomethane.

Both carbene and ketone groups can react with -OH and -SH groups, and possibly -NH groups on vacuolar proteins. Carbenes can easily be inserted to these bonds. Ketenes can react with these nucleophiles to give esters, thioesters, or amides. When vacuolar membrane vesicles were labeled with radioactive NBZ arginyl diazomethane by UV irradiation and then treated with base-hydroxylamine or hydroxide, radioactivity associated with proteins disappeared completely (Table 4). This result suggested that the probable binding site

Table 4. *Base treatment of labelled vacuolar proteins.* Vacuolar proteins (20 μg) were labelled with NBZ arginyl diazomethane (0.4 μM, 50 Ci/mmol) by incubation for 10 min at 30 °C and then treated with base. Concentrated trichloroacetic acid was then added to the reaction mixture and radioactivities remaining with protein precipitates were measured as described in Materials and Methods.

Treatment	Precipitate
	(cpm)
None	1920
2 M NH_2OH	0
1 M NaOH	0

[a] Background (0 min incubation with radioactive NBZ arginyl diazomethane) was subtracted.

is -OH or -SH group and the reaction was accomplished by ketene group which was formed via fast intramolecular rearrangement -i.e. the Wolff rearrangement.

Treatment with cysteinyl blocking reagent.

If -SH group of cysteine in vacuolar protein were involved in labeling, labeling would be abolished by cysteinyl blocking reagents-i.e. Iodoacetic and NEM. When vacuolar proteins were incubated with iodoacetic acid or NEM and then irradiated with a UV lamp in the presence of radioactive NBZ arginyl diazomethane, radioactivity associated with proteins decreased (Table 5). This result suggested that cysteine may be the labeling site.

Table 5. *Labelling vacuolar proteins with radioactive NBZ arginyl diazomethane after treatment with cysteinyl blocking ragents.*
Vacuolar proteins (20 μg) were treated with 10 mM iodoacetic acid or 10 mM NEM and then irradiated with UV in the presence of radioactive NBZ arginyl diazomethane (0.4 μM, 50 Ci/mmol) as described in Materials and Methods. After irradiation, radioactivity bound to protein precipitates was measured as described in Materials and Methods.

Treatment	Radioactivity
	(%)
None	100
Iodoacetic acid	36
NEM (N-Ethyl-Maleimide)	50

DISCUSSION

To identify and purify the arginine transporter, there are several possible approaches. One approach is the solubilization of the transporter protein with detergent, fractionation of proteins and, reinsertion into a lipid bilayer. However, the amount of transporter proteins in cell is often very low, detergent treatment can separate substrate-receptor complexes, and the substrate binding activity might be lost after solubilization in detergents and may not be regained by insertion into lipid bilayer. Instead of using the arginine binding activity to identify the arginine transporter, labeling the protein with an affinity label was chosen. Phoaffinity labeling has been used widely to study a variety of biological systems (Bayley and Knowels, 1976; Chowdhry and Westheimer, 1979). It has been used to study the active site of enzymes (Escher and Schwyzer, 1974; Chen and Guillory, 1979) for the identification of enzymes (French, 1963) and transporter (Knowles, 1972; Staros and Knowles, 1978; Hourk *et al.*, 1983; Hourk and Wright, 1983; Yurknow and Laskin, 1987). Since the arginine transporter is not expected to have catalytic functions, photoaffinity label was sought which would be recognized by the arginine transporter and become attached to the protein by itself.

Among several arginine derivatives, NBZ arginyl diazomethane was chosen as a possible label for arginine transporter. Since the diazo compound is unstable and easily undergoes the Wolff rearrangement, NBZ arginyl diazomethane has not previously been used for affinity labeling. Also it is known to be difficult to synthesize the arginine derivative due to amino groups with similar reactivity. However, this derivative has an intact guanido side chain and L-configuration, which should make it recognizable by the arginine transporter (Paek and Weiss, 1989), and has a diazo group which becomes reactive upon UV or heat treatment (Smith and Dieter, 1981). Its reactivity is masked in the absence of UV light or heat avoiding conditions. After it is recognized by the arginine transporter, it can be attached to the arginine transporter by the carbene group produced form

the diazo group by UV light or heat treatment. So, it appeared to be the most suitable analog for labeling the arginine transporter protein. In addition to this advantage, it is stable for several months as lyophilized powder and the yield of synthesis was more than 60%.

Using NBZ arginyl diazomethane, vacuolar arginine transporter was identified (Paek and Weiss, 1989). This arginyl derivative inhibited arginine transport very well at low concentrations. L-Arginine transport was 6.8% in the presence of this derivative compared to 18% in the presence of L-arginine. Better inhibition on arginine transport by NBZ arginyl diazomethane than L-arginine suggested the suicidal irreversible binding of NBZ arginyl diazomethane to the arginine transporter blocking the reversible binding of L-arginine. Irreversible binding was clearly observed when arginine transport was completely inhibited when vacuoles were preincubated even at 1 mM of this derivative.

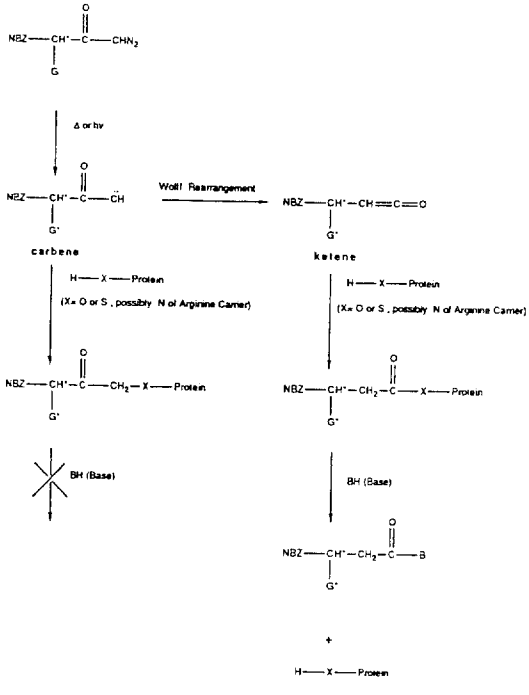

Fig. 2. *The possible reaction pathways of attachment and detachment of radioactive NBZ arginyl diazomethane to the argining transporter.*
The *shows the positions of radioactivity. Abbreviations are: NBZ, N-*p*-Nitrobenzoxycarbonyl; G, guanido side chain.

Covalent binding of this derivative can be accomplished by carbene group and ketene group which is produced from the carbene by the Wolff rearrangement. The possible mechanisms of binding through the carbene and ketene groups are shown in Fig. 2. Carbene group is very reactive and can be inserted into many different kinds of bonds. Compared to this, binding through ketone group will give more specific labeling to -OH or -SH groups on proteins. Since the labeled derivative was detached by strong nucleophiles, the bond between the arginine derivative and the arginine transporter is likely to be an ester bond which could be formed by the attack of nucleophilic group such as -OH or -SH of the arginine transporter on the ketene rather than insertion of carbene group between heteroatoms. Since the cysteinyl blocking group inhibited the labeling, the -SH of cysteine might attack the ketene group forming thioester bond. Cysteine has been suggested to be involved in the transport mechanism for basic amino acids in yeast vacuoles (Ohsumi and Anraku, 1981). Up until now, carbene has been known to be produced from diazo group only by UV irradiation or high temperature (higher than 60°C). So it was a surprise that NBZ arginyl diazomethane could be attached even at 30°C. To find out the exact mechanism of binding and the binding site, more work needs to be done.

Although both ATP-dependent and independent arginine transport in *N. crassa* were inhibited in the presence of this arginyl derivative, only one protein was labeled (Paek and Weiss, 1989). One kind of protein may mediate two different mechanisms of L-arginine transport-passive and ATP mediated activity transport having two functions-recognition and transport. However it is not known how vacuolar ATP hydrolysis is related to L-arginine transport. More works should be done to find out the exact mechanism of L-arginine transport.

적 요

액포에 존재하는 arginine 운반체의 인식 특이성에 근거하여 NBZ arginyl diazomethane을 합성, affinity label로 사용하였다. 이 arginyl derivative는 ATP-dependent와 ATP-indepndent에 의한 arginine 운반작용을 억제하였다. 액포에의 결합은 비역가적이며, 강한 염기에 의해 분리되었다. Cysteine을 blocking시키면, 결합은 일어나지 않았다.

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