is the elucidation of the specific protein ligands to the zinc atoms and how the zinc atoms participate in the catalytic reaction and in the protein structure.

Acknowledgments

We thank the Department of Pharmacology and Toxicology for the use of the Instrumentation Laboratories atomic absorption spectrophotometer and the Department of Physiology and Biophysics for the use of the Nuclear Chicago γ counter.

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Possible Site-Specific Reagent for the General Amino Acid Transport System of *Saccharomyces cerevisiae*†

Fred S. Larimore and Robert J. Roon*

ABSTRACT: The general amino acid transport system of *Saccharomyces cerevisiae* functions in the uptake of neutral, basic, and acidic amino acids. The amino acid analogue *N*-δ-chloroacetyl-L-ornithine (NCAO) has been tested as a potential site specific reagent for this system. L-Tryptophan, which is transported exclusively by the general transport system, was used as a substrate. In the presence of glucose as an energy source, NCAO inhibited tryptophan transport competitively ($K_i = 80\ \mu M$) during short time intervals (1–2 min), but adding 100 μM NCAO to a yeast cell suspension resulted in a time-dependent activation of tryptophan transport during the first 15 min of treatment. Following the activation a time-dependent decay of tryptophan transport activity occurred. Approximately 80% inactivation of the system was observed after 90 min. When a yeast cell suspension was treated with NCAO in the absence of an energy source, an 80% inactivation of tryptophan transport occurred in 90 min. The inactivation was noncompetitive ($K_i \simeq 60\ \mu M$) and could not be reversed by the removal of the NCAO. Addition of a fivefold excess of L-lysine during NCAO treatment or prevented inactivation of tryptophan transport. Under parallel conditions of incubation, other closely related transport systems were not inhibited by NCAO.

The transport of amino acids in *Saccharomyces cerevisiae* is accomplished by a general amino acid transport system having a broad substrate specificity and by a number of specific transport systems having more stringent substrate specificities (Crabeel and Grenson, 1970; Gits and Grenson, 1967; Grenson et al., 1966, 1970; Jorris and Grenson, 1969). The maximal level of activity observed with the general amino acid transport system is 5- to 50-fold higher than that found with the various specific systems. Grenson and her associates, who initially

† From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received August 1, 1977.* This work was supported in part by Research Grant No. GM 23135 from the National Institutes of General Medical Sciences.

0006-2960/78/0417-0431$01.00/0

identified the general system, reported that neutral and basic L-amino acids were good substrates (Grenson et al., 1970). Subsequent studies in this same laboratory indicated that the general system functions in the uptake of acidic L-amino acids as well (Darte and Grenson, 1975). Recently, Rytka demonstrated that D-amino acids are also transported by the general system (Rytka, 1975).

The kinetic characteristics of the general amino acid transport system suggest that it should provide an excellent model for the study of amino acid transport in the eukaryotic cell (Grenson et al., 1970). In addition to the well-known technical and genetic advantages of *Saccharomyces*, the general system offers two specific advantages to the researcher. (1) It is relatively active compared with other transport systems of *Saccharomyces*, suggesting that a considerable amount of transport (binding) protein might be present. (2) Its broad substrate specificity and high affinity for its better substrates ($1-5 \times 10^{-6}$ M) indicate that there may be a good potential for labeling a transport-associated binding protein with an affinity amino acid and/or for purifying a binding protein using an amino acid affinity column.

Preliminary studies in our laboratory (R. Roon, unpublished data) indicate that unsubstituted α-amino and α-carboxyl moieties are necessary for substrate binding to the general amino acid transport system but that there is a wide latitude with respect to the size and orientation of the amino acid side chain. With the exception of acidic amino acids with their side chain carboxyls in the unprotonated form, all the α-amino acids tested seemed to have a high affinity for the system. The implication of these findings is that affinity reagents directed at the binding site of the general transport system should have unsubstituted α-amino and α-carboxyl groups and should carry a neutral or basic side chain. In the present communication we wish to report the effect of one such potential site specific reagent, δ-*N*-chloroacetylornithine (NCAO),[1] on the activity of the general amino acid transport system.

Materials and Methods

Organisms. The haploid yeast strain X2180-1A (*a*, Suc 2, mal, gal 2), was obtained from the Yeast Genetics Stock Center, University of California, Berkeley. This strain was used in all experimental procedures except for a single designated control experiment. Mutant strain AR102 (*a*, Suc 2, mal, gal 2, gap 1) was derived from strain X2180-1A by methodology that has been reported elsewhere (Dunlop and Roon, 1975).

Growth Conditions. The standard chemically defined medium contained, per liter: 20 g of D-glucose, 2 g of yeast nitrogen base (Difco no. 033-15-9, without amino acids and ammonium sulfate), and 1.32 g of ammonium sulfate. Yeast cultures were grown at 23 °C with slow rotary shaking at 150 rpm from a 1% inoculum. The cells were harvested by centrifugation after approximately 16 h of growth. (At the time of harvesting the absorbance of the cell suspension at 660 nm was ~1.0 using cuvettes with a 10-mm light path.) The harvested cellular material was used immediately for the assay of amino acid or methylamine transport activity.

Assay of the General Amino Acid Transport System. Tryptophan was selected as the substrate for assaying the general amino acid transport system because it is transported exclusively by this system and exhibits a low K_m (~4 μm) and a relatively high V_{max} (20 nmol per min per mg of cells). For the assay of tryptophan transport, cells were suspended to 0.5 A_{660} in medium containing a 3% D-glucose and 20 mM potassium phosphate buffer, pH 6.5 (phosphate–glucose buffer), and incubated at 23 °C with slow shaking (150 rpm). After 2–4 h of incubation 4.5-mL samples were removed and mixed with 0.5 mL of 1 mM L-[^{3}H]tryptophan (specific activity $\simeq 1.0 \times 10^3$ counts per min per nmol). Samples (1.0 mL) of this mixture were removed at 30-s intervals for 2 min, poured onto membrane filters (Gelman filters, 25-mm diameter, 0.45-μm pore size), and immediately washed with 10 mL of ice-cold water. The washed filters were placed in vials containing 5 mL of liquid scintillation counting fluid.

Assay of Methylamine Transport. Cells were suspended to 1.0 A_{660} in medium containing 2% D-glucose and 10 mM potassium phosphate buffer, pH 6.7, and incubated at 23 °C with slow shaking (150 rpm). After 90–180 min, 4.5-mL samples were removed and mixed with 0.5 mL of 5 mM [^{14}C]methylamine (specific activity = 5×10^5 counts per min per μmol). Samples (1.0 mL) of this mixture were removed at 1-min intervals for 4 min, poured onto membrane filters (Gelman filters, 25-mm diameter, 0.45-μm pore size), and immediately washed with 10 mL of ice-cold water. The washed filters were placed in vials containing 5 mL of liquid scintillation counting fluid.

Determination of Radioactivity. The scintillation fluid for determination of ^{3}H-labeled amino acids and [^{14}C]methylamine contained 9.9 g of 2,5-diphenyloxazole, 609 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 3000 mL of toluene, and 390 mL of Bio-Solv BBS-3 (obtained from Beckman Instruments, Inc.). Radioactivity was measured on a Beckman LS100 liquid scintillation counter.

Transport Activities. All uptake velocities are expressed as nmol of substrate transported per min per mg of cells (dry weight).

Chemicals. The radiochemicals used in this study were purchased from Amersham/Searle Corp. All other chemicals were of reagent grade and obtained from commercial sources.

Synthesis and Characterization of δ-N-Chloroacetylornithine. L-Ornithine·HCl (4.0 g) was dissolved in 20 mL of water. The solution was heated to 100 °C and 20 mL of an aqueous solution containing 11.0 g of $CuCO_3$ (Green-Basic) was added dropwise with constant stirring. The solution was cooled to 25 °C in an ice bath and filtered. NaOH (5 mL of a 4 N solution) was added and the solution was cooled to 4 °C in an ice–water bath. Chloroacetic anhydride (5.1 g) was added at 4 °C over a 30-min period. The pH was maintained above 10 by the addition of 4 N NaOH during the reaction with chloroacetic anhydride. The solution was then warmed to 25 °C and allowed to stand for 30 min. Concentrated HCl was added dropwise with constant stirring to lower the pH to 2.0. Hydrogen sulfide gas was bubbled through the solution for 10 min and the solution was filtered. The filtrate was concentrated in vacuo to 15 mL and passed over a column of Dowex 50W, 400 mesh (2.5 × 18 cm), which had been previously equilibrated with 75 mM ammonium acetate. Under these conditions NCAO is eluted from the column with the initial wash, whereas L-ornithine is retarded. The column eluate containing NCAO was dried in vacuo. The product, NCAO, was suspended in a minimal volume of water at 50 °C, a few drops of absolute ethanol were added, and crystallization was allowed to proceed at room temperature. The recrystallized product (mp 195 °C) gave a single ninhydrin-positive spot on thin-layer chromatography, R_f 0.47, on cellulose acetate plates with *N*-butyl alcohol:water:acetic acid (4:1:1 v/v) as solvent, and also gave a single ninhydrin-positive peak on the Beckman amino acid analyzer, Model 120C. The amino acid analysis was conducted with the standard Beckman analyzer buffers

[1] Abbreviation used: NCAO, δ-*N*-chloroacetylornithine.

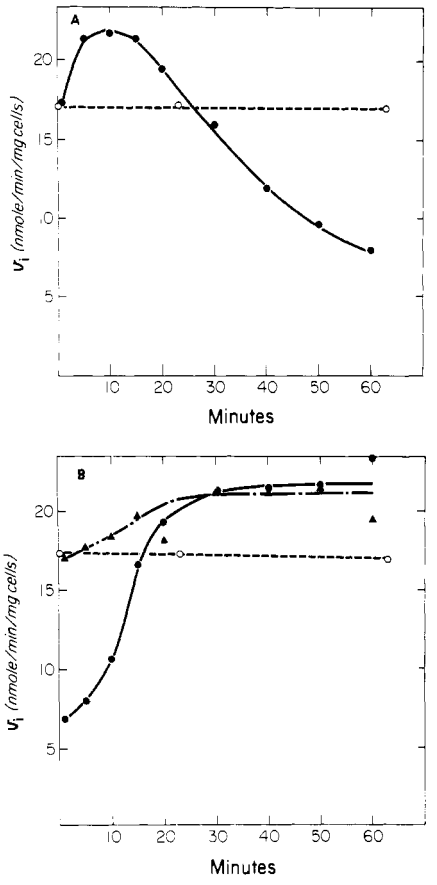

FIGURE 1: Time-dependent alterations of tryptophan transport activity. (A) Effect of NCAO addition in the presence of an energy source. A yeast cell suspension was incubated in phosphate-glucose buffer for 2 h. The cell suspension was then divided into two portions. NCAO, 100 μM final concentration, was added to one portion (●—●) and the other served as a control (○—○). Tryptophan transport activity was determined at 10-min intervals under standard assay conditions. (B) Effect of ornithine and glutamate addition in the presence of an energy source. Tryptophan transport activity was assayed under conditions given in A. No addition (○- - -○); 100 μM L-ornithine (●—●); 100 μM L-glutamate (▲- - -▲).

on a 54-cm AA15 resin column. The NCAO peak appeared in the middle of buffer 2, with a retention time of ~57.7 min, while the ornithine peak appeared in buffer 3 at ~146.8 min. Analytical results with NMR, IR, and mass spectroscopy were consistent with the proposed structure. Elemental analysis was performed by Galbraith Laboratories, Inc., and gave the following results. Anal. Calcd: C, 40.29; H, 6.24; N, 13.43; O, 23.02; Cl, 17.03. Found: C, 40.25; H, 6.30; N, 13.43; O, 22.92; Cl, 17.18.

Reaction of NCAO with Yeast Cell Suspensions in Phosphate-Sorbitol Buffer. Cells were suspended to 0.5 or 1.0 A_{660} in medium containing 3% D-glucose and 20 mM potassium phosphate buffer, pH 6.5, and incubated at 23 °C with slow shaking (150 rpm). After 2-3 h of incubation, the cells were harvested by centrifugation and suspended to 0.5 or 1.0 A_{660} in medium containing 3% sorbitol and 20 mM potassium phosphate buffer, pH 6.5 (phosphate-sorbitol buffer). After 10 min the reaction was initiated by the addition of NCAO at the levels indicated. Cell samples (4.5 mL) were removed at the times indicated, collected on membrane filters (Gelman Filters, 25-mm diameter, 0.45-μm pore size), and washed with 20 mL of phosphate-sorbitol buffer. The washed cells were suspended in 4.5 mL of phosphate-glucose buffer and after 10 min were assayed for tryptophan or methylamine transport activity in the absence of NCAO.

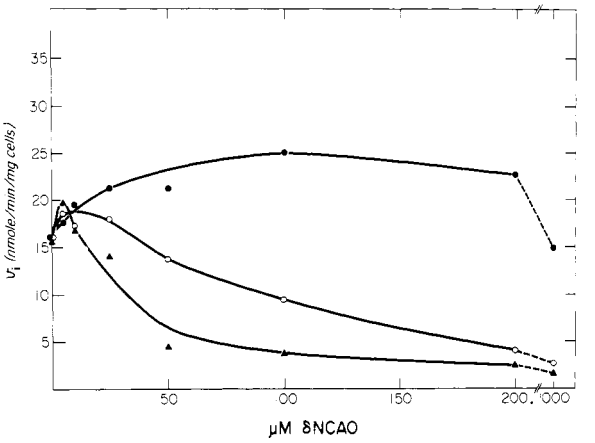

FIGURE 2: Tryptophan transport activity as a function of NCAO concentration in the presence of an energy source. Tryptophan transport activity was assayed under conditions given in Figure 1A in the presence of varying concentrations of NCAO after 15 (●—●), 60 (○—○), and 120 (▲—▲) min of treatment.

Results

Effect of NCAO on the General Amino Acid and Methylamine/Ammonia Transport Systems: Nonspecific Effects in the Presence of an Energy Source. General Amino Acid Transport System. The effect of NCAO on tryptophan transport activity was determined using a culture of *Saccharomyces* suspended in 20 mM potassium phosphate buffer, pH 6.5, containing 3% glucose as an energy source. Under these conditions the general amino acid transport system is highly active. Figure 1A shows the effect of 100 μM NCAO on tryptophan transport activity. The rate of transport was initially unaffected by the addition of NCAO; transport activity increased approximately 20% during the first 10 min of incubation followed by a time-dependent inactivation. After 60 min, transport activity had fallen to approximately 50% of normal levels. As a control, ornithine, a high affinity substrate for the general amino acid transport system ($K_m \sim 4$ μM at pH 6.0), and glutamate, a low affinity substrate ($K_m \sim 1$ mM at pH 6.0), were tested as inhibitors of tryptophan transport. As shown in Figure 1B, ornithine (100 μM) produced an initial competitive inhibition of tryptophan transport followed by a time-dependent activation of ~20%. Under the assay conditions used here, the extracellular ornithine pool should have been completely depleted after ~30 min. When 100 μM L-lysine was substituted for L-ornithine, a similar pattern of inhibition, followed by activation, was observed (not shown). Glutamate (100 μM) addition produced no initial inhibition but resulted in a time-dependent activation of ~20%.

These data suggested that the NCAO dependent activation of tryptophan transport was a nonspecific effect that could be mimicked by any transportable amino acid, but that the subsequent time-dependent inactivation might be related to the ability of NCAO to serve as an alkylating agent. The lack of initial inhibition by NCAO (under these assay conditions) further suggested that the reagent had a substantially lower affinity for the general amino acid transport system than either tryptophan, ornithine, or lysine.

The effect of NCAO concentration on tryptophan transport was examined after 15, 60, and 120 min of treatment (Figure 2). Activation occurred at all but the highest (1 mM) levels of reagent during the first 15 min whereas after 120 min inhibition was evident at all NCAO levels above 10 μM. Under these conditions, a half-maximal rate of inactivation occurred in the range of 50-100 μM NCAO.

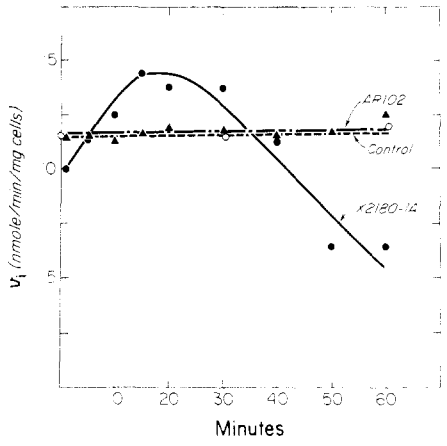

FIGURE 3: Time-dependent alteration of methylamine transport activity; effect of NCAO addition in the presence of an energy source. A cell suspension of wild type yeast strain X2180-1A was incubated in phosphate-glucose buffer for 2 h. The cell suspension was divided into two portions. NCAO, 100 μM final concentration, was added to one portion (●—●) and the other served as control (○- - -○). Methylamine transport activity was determined at 10-min intervals under standard assay conditions. Yeast strain AR102, which lacks activity for the general amino acid transport system, was assayed in the presence of 100 μM NCAO under similar conditions (▲—▲).

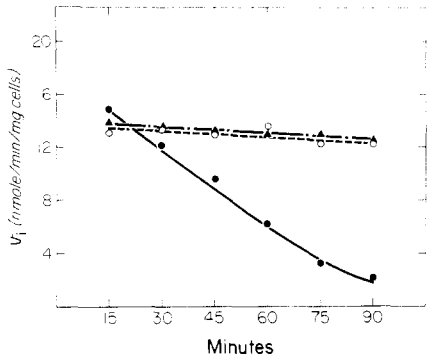

FIGURE 4: Time dependency for NCAO inhibition in the absence of an energy source; effect on tryptophan transport activity. Yeast cell suspensions were incubated in phosphate-sorbitol buffer and assayed at the times indicated (as outlined under methods): 100 μM NCAO (●—●), 100 μM NCAO + 500 μM L-lysine (▲—▲), control (○- - -○).

Methylamine/Ammonia Transport Systems. In order to examine the specificity of NCAO inhibition, the effect of the reagent on methylamine transport was examined. Methylamine is transported in *Saccharomyces* by a specific transport system which has a high affinity for ammonia and methylamine but does not transport amino acids (Roon et al., 1975a,b). The methylamine/ammonia transport system was selected as a control in this study because its sensitivity to ionophores, uncouplers, ATPase inhibitors, and alkylating agents is similar to the general amino acid transport system (Roon et al., submitted for publication). The present evidence suggests that methylamine/ammonia and amino acid transport systems may share common energy coupling and/or translocation components but probably have unique binding proteins.

As shown in Figure 3, in phosphate-glucose buffer NCAO affected methylamine transport in a manner analogous to its action on tryptophan transport. Activation was observed at short time intervals followed by a time-dependent inactivation which reached ~50% after 60 min. In a transport deficient mutant, AR102 (gap 1), which lacks the general amino acid transport system, methylamine transport was completely insensitive to NCAO.

These data indicated that, in the presence of glucose as an energy source, NCAO was not serving as a site specific reagent for the general amino acid transport system. Rather, the evidence suggested that NCAO was being transported into the cell where it inhibited transport activity in a nonspecific manner.

Effect of NCAO on the General Amino Acid and Methylamine/Ammonia Transport Systems: Specific Effects in the Absence of an Energy Source. General Amino Acid Transport System. It seemed possible that the specificity for NCAO inhibition of the general amino acid transport system might be enhanced under conditions which did not permit the active transport of amino acids into the cell. The assumption made was that in the absence of an energy source, NCAO would still be able to interact with externally directed binding sites on the general amino acid transport system but would not be concentrated within the cell. In order to test this, 3% sorbitol was substituted for glucose in the reaction medium. At this level sorbitol seems to serve as an osmotic stabilizer for yeast cell suspensions but it is not utilized as an energy source. The rate of amino acid transport in the presence of sorbitol is equivalent to that observed in the absence of any energy source, i.e., less than 5% of normal transport activity (F. Larimore, unpublished data).

Figure 4 shows the time courses for inhibition of tryptophan transport by 100 μM NCAO in phosphate-sorbitol buffer. After 90 min of treatment with NCAO, transport activity was inhibited ~80%. When 500 μM L-lysine was present in the reaction medium, no inhibition was observed. Since lysine has a high affinity for the general amino acid transport system (Grenson et al., 1970), its protective effect is consistent with the site specific nature of NCAO inhibition. Under similar incubation conditions addition of 1 mM methylamine, 1 mM dithiothreitol, or 5 mM mercaptoethanol did not reduce the extent of NCAO inhibition (not shown).

Methylamine/Ammonia Transport System. The methylamine/ammonia transport system was uneffected by 100 μM NCAO in phosphate-sorbitol buffer (see Supplementary Material Available paragraph).

Reaction Kinetics for NCAO Inhibition of the General Amino Acid Transport System in the Absence of an Energy Source. Figure 5 gives the pH profile of NCAO inhibition in phosphate-sorbitol buffer. At pH values below 7.0, NCAO was an effective inhibitor of tryptophan transport. However, above pH 7.0 there was a dramatic decrease in the effectiveness of NCAO as an inhibitor. A decrease in the amount of inhibition was also observed below pH 4.0. In other experiments (not shown) we have found that the transport of neutral amino acids by the general amino acid transport system is most rapid between pH 4.0 and 7.0. Therefore it seems possible that the decrease in NCAO inhibition below pH 4.0 and above pH 7.0 could be related to the binding characteristics of the transport system.

An important criterion for an affinity reagent is the formation of an irreversible complex with the protein. The next phase in the characterization of NCAO inhibition was to determine whether the inhibition was reversible or irreversible. The cells were pretreated with NCAO in the phosphate-sorbitol buffer; then they were washed and resuspended in the phosphate-glucose buffer. Transport was assayed in the presence and absence of cycloheximide. In the absence of cycloheximide a small time-dependent increase (Δ of ~2 nmol per min per mg of cells) in transport activity was observed. In the presence of cycloheximide, no increase was observed (Supplementary Material). These results were indicative of the inhibition being irreversible; the increase in transport ac-

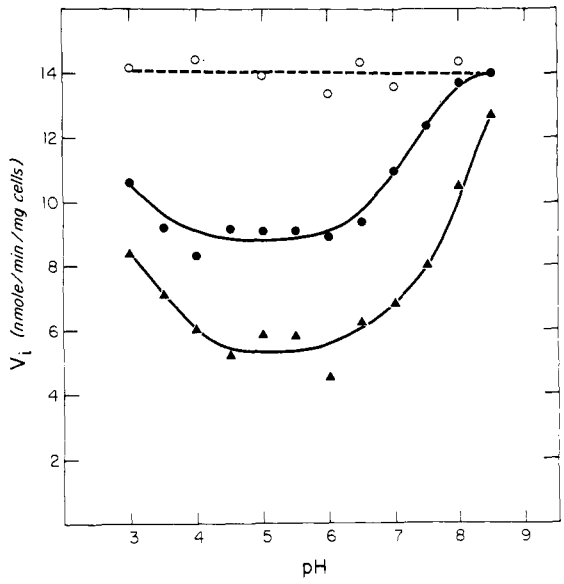

FIGURE 5: pH profile for the inhibition of tryptophan transport by NCAO in the absence of an energy source. Yeast cell suspensions were incubated with 100 μM NCAO in phosphate-sorbitol buffer for 30 min (●-●) and 60 min (▲—▲) at the pHs indicated. Control suspensions were treated in a parallel fashion in the absence of NCAO (O- - -O). Tryptophan transport activity was determined under standard assay conditions as indicated under methods.

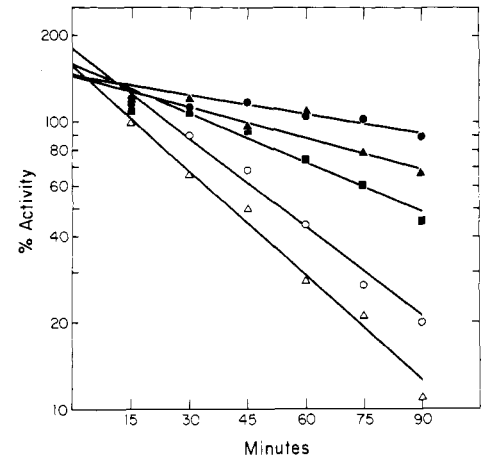

FIGURE 6: Kinetics of inhibition of tryptophan transport by NCAO in the absence of an energy source. Tryptophan transport activity was determined after incubation of cell suspensions in phosphate-sorbitol buffer with 5 μM (●—●), 10 μM (▲—▲), 25 μM (■—■), 50 μM (O—O), and 100 μM (△—△) NCAO.

tivity in the absence of cycloheximide could be attributed to the de novo synthesis of transport proteins.

If NCAO is a site-specific affinity reagent, it should show an initial competitive inhibition and a time-dependent noncompetitive inhibition of tryptophan transport. When transport was assayed in the phosphate-glucose buffer immediately after the addition of NCAO, competitive inhibition was observed. The K_i for NCAO was ~80 μM. However, when the cells were treated with NCAO in phosphate-sorbitol buffer for 45 min prior to assaying for transport, noncompetitive inhibition was found (Supplementary Material).

In Figure 6 the tryptophan transport activity is plotted logarithmically as a function of time at various concentrations of NCAO. From these data the K_i for the noncompetitive inhibition of tryptophan transport can be calculated by the method of Kitz and Wilson (1962). The slopes of each line give a k_{app}, where

$$k_{app} = \frac{k_3(I)}{I + K_i}$$

and k_3 is the rate constant for the conversion of EI, the reversible inhibitor-enzyme complex to EI′, the irreversible inhibitor-enzyme complex according to the equation

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} EI'$$

K_i was determined from a double-reciprocal plot of $1/k_{app}$ vs. $1/I$ in which the slope equals K_i/k_3 and the intercept with the abscissa equals $1/k_3$ (data not shown). By this method, a K_i of 60 μM was determined for the inhibition of tryptophan transport.

Discussion

The experimental evidence of this study suggests that NCAO has a high affinity for the substrate binding site of the general amino acid transport system and that, in the absence of a cellular energy source, it may serve as a highly specific site-directed reagent.

These data further suggest that radiolabeled NCAO may be of use in irreversibly labeling, purifying, and characterizing the substrate binding component of the general amino acid transport system. Although this approach has the inherent limitation of producing an inactive product, it could prove useful if the isotopically labeled binding component could be copurified with unlabeled material. In the event this does not occur, purified fractions of the inactive modified material might be of use in raising antibodies to the native binding component.

Radiolabeled NCAO may also be useful in studying transport kinetics and possibly might provide a method for quantitating the number of amino acid binding sites of the amino acid transport system. This latter approach would be of particular use in determining whether changes in the number of amino acid transport binding sites occur in response to nitrogen catabolite repression (Roon et al., 1975).

Acknowledgments

We wish to thank Ms. Alicia Chan for her technical assistance, Mr. Tim Church for his help with computer programming, and the University of Minnesota for supplying computer time at the Health Sciences Computer Center.

Supplementary Material Available

The effects of NCAO on methylamine/ammonia transport in phosphate-sorbitol buffer, the effects of cycloheximide on NCAO-treated cells in phosphate-glucose buffer, and the Lineweaver-Burk plot of NCAO inhibition are presented in three figures (6 pages). Ordering information is given on any current masthead page.

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Hydrolysis of Proteins Using Dipeptidyl Aminopeptidases: Analysis of the N-Terminal Portion of Spinach Plastocyanin†

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ABSTRACT: The exopeptidases dipeptidyl aminopeptidases I and IV were used to hydrolyze the N-terminal portion of spinach plastocyanin to dipeptides. The enzymes were used individually as well as in a mixture and the dipeptides were analyzed by combined gas chromatography–mass spectrometry. Data are presented for native plastocyanin and the S-methylated protein. Of the 98 residues which make up this protein, the first 44 were released in the form of 22 dipeptides by the combined action of DAP I and DAP IV. These dipeptides were aligned by homology to other plastocyanins of known sequence. The results demonstrate the versatility of the two enzymes in hydrolyzing proteins to obtain information on their primary sequence.

Gutman and Fruton (1948) first described a chymotrypsin-like proteolytic enzyme isolated from bovine spleen which catalyzed the deamidation of hydrophobic dipeptide amides. This enzyme was later termed cathepsin C (Tallan et al., 1952). Several papers concerned with the properties of this enzyme were then published (Izumuja and Fruton, 1956; Metrione et al., 1966) which concluded that the enzyme had extremely limited substrate specificity. McDonald et al. (1965) published the first of a series of papers investigating the properties of a chloride-activated sulfhydryl-dependent enzyme from bovine pituitary gland similar in action to bovine spleen cathepsin C. This enzyme was later found (McDonald et al., 1969) to catalyze the sequential release of dipeptides from polypeptides and was termed dipeptidyl aminopeptidase I (EC 3.4.14.1, DAP I).[1] Subsequent work with the spleen cathepsin C lead to the conclusion that it was identical with the pituitary enzyme (McDonald et al., 1972). With the necessary activators it was found that DAP I showed a very broad substrate specificity in that it was able to hydrolyze dipeptides from the N termini of β-corticotropin, glucagon, secretin, oxidized bovine insulin B-chain, and angiotensin-II. The studies showed that all unsubstituted amino-terminal dipeptidyl groups can be hydrolyzed by DAP I with the exception of those in which arginine or lysine is the N-terminal residue or which involve hydrolysis of a peptide bond involving proline (McDonald et al., 1971).

The use of DAP I in sequence studies was first proposed by McDonald et al. (1969) where they utilized paper chromatography to identify the dipeptides released in the time course reaction for hydrolysis of several small polypeptides. Subsequent work by Callahan et al. (1972) applied several physical methods for the separation and identification of the released dipeptides, including paper chromatography, ion-exchange chromatography, thin-layer chromatography on both polyamide and microcrystalline cellulose, and membrane diffusion.

Other enzymes having specificities much narrower in scope than DAP I have been isolated from a number of mammalian tissues. Hopsu-Havu and Sarimo (1967) isolated an enzyme from rat liver and hog kidney which hydrolyzed Gly-Pro-β-naphthylamide. Based on the substrate specificity reported for this enzyme, McDonald et al. (1971) named the enzyme dipeptidyl aminopeptidase IV (DAP IV). DAP IV preferentially hydrolyzes peptide bonds in dipeptide β-naphthylamides and small peptides in which proline is in the penultimate position, although it is not limited to these substrates. Caprioli and Seifert (1975) showed that DAP IV will also hydrolyze dipeptides from polypeptides containing arginine and lysine in the N-terminal positions, but will not hydrolyze the imide bond of a prolyl residue.

The use of a mixture of DAP I and DAP IV for the hydrolysis of polypeptides was first demonstrated by Caprioli and Seifert (1975). Since the two enzymes have somewhat complementary activities, the mixture provides a convenient way of circumventing many of the specificity problems associated with either enzyme, except that of the prolylimide bond.

Although many analytical methods have been employed to identify the dipeptides, the most effective method involves their simultaneous separation and identification utilizing combined gas chromatography/mass spectrometry (GC/MS). The use of GC/MS techniques in combination with DAP I hydrolyses was reported earlier by Ovchinnikov and Kiryushkin (1972) and Caprioli et al. (1973). Subsequent work by Caprioli and Seifert (1975) demonstrated the versatility and reliability of

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[1] Abbreviations used: DAP, dipeptidyl aminopeptidase; GC/MS, combined gas chromatography/mass spectrometry; Pth, phenylthiohydantoin.

0006-2960/78/0417-0436$01.00/0