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Photoaffinity labeling and characterization of the cloned purine-cytosine transport system in *Saccharomyces cerevisiae*

(8-azidoadenine/molecular cloning/membrane protein/glycosylation)

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ABSTRACT **8-Azido[2-^{3}H]adenine was used as a photoaffinity label for the purine-cytosine transport system. After irradiation in the presence of the photoaffinity label, the cells were converted into protoplasts, their plasma membranes were purified, and the membrane proteins were extracted and separated by NaDodSO$_4$/PAGE. The radioactivity was specifically incorporated into a protein with a molecular weight of 120,000. Photoaffinity labeling of this protein could be blocked by irradiation in the presence of natural substrates for the transport system. The molecular weight as determined by NaDodSO$_4$/PAGE was found to be twice the value calculated from mRNA analysis of the cloned gene. Incubation of exponentially growing cells with tunicamycin, an antibiotic that inhibits glycosylation of proteins, resulted in a 40% decrease in the overall initial uptake rate, which correlates with the reduction of the labeled M_r 120,000 protein. Treatment of the extracted labeled plasma membrane proteins with glycosidic enzymes resulted in disappearance of the M_r 120,000 peak and the appearance of new peaks at M_r 60,000 and M_r 73,000. These findings indicate that the purine-cytosine transport protein is a glycoprotein.**

The uptake of the purine bases adenine, guanine, and hypoxanthine and the pyrimidine base cytosine in *Saccharomyces cerevisiae* is catalyzed by a single energy-dependent transport system (1, 2). *In vivo* properties of the transport system such as genetic characterization, energy coupling, and a possible mechanism of substrate binding to the purine-cytosine transport system are described in refs. 3–6.

For the further characterization of the transport system, we found 8-azidoadenine to be a suitable photoaffinity label. It has a high affinity (K_m = 9 × 10^{-6} M) without being translocated and it is bound covalently to the transport protein on irradiation with UV light. Its synthesis and properties are described in ref. 7.

The purine-cytosine transport system was cloned on a multicopy plasmid that can replicate autonomously in yeast or in *Escherichia coli*. Cloning was necessary to (*i*) increase the amount of transport protein in the yeast plasma membrane, (*ii*) allow measurement of the length of the mRNA transcript of the cloned gene, and (*iii*) enable sequencing of the gene.

In this work, we used 8-azido[2-^{3}H]adenine as a photoaffinity label. The transport protein was labeled *in vivo*, the plasma membranes of the labeled cells were purified by using cationic silica microbeads (8), and the plasma membrane proteins were extracted for further characterization.

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MATERIALS AND METHODS

Strains and Culture Conditions. *S. cerevisiae* RXII (a gift of A. Kotyk, Prague) was used as the diploid wild-type strain. The mutant strain NC117spl (*α leu2 ade1 fcy2-3*) was obtained by a cross of NC99spl (*a ade1 fcy2-3*) (a derivative of FL100; ATCC 28383) × GRF18 (*his3 leu2*). The strains were cultivated in GYNP medium (2% glucose/1% Difco yeast nitrogen base/0.5% peptone) containing hypoxanthine at 10 μg/ml at 30°C with agitation.

When cells from the stationary growth phase were prepared for maximum uptake, a 50-ml culture was preincubated in 250 ml of glucose/citrate buffer (50 mM sodium citrate, pH 5.5/2% glucose) at 30°C under aeration for 60 min.

To transform *E. coli*, strain BJ5183 (F$^-$ *recBC sbcB endoI gal met str hsdR thi bio*) was used as receptor strain.

Cloning Procedure. Cloning was accomplished by using two yeast gene libraries, the construction of which is described by Losson and Lacroute (9). The plasmid used as a vector was pJDB207. In addition to two distinct origins of DNA replication, one of which is functional in *E. coli* and the other, in yeast, it contains the yeast *LEU2* gene and the genes conferring ampicillin and tetracycline resistance to *E. coli* (10). The yeast strain NC117spl (*α leu2 ade1 fcy2-3*) was used as recipient in yeast transformation. Because of their *fcy2* mutation, *ade*$^-$/*leu*$^+$-transformed cells (except those having recovered a functional purine-cytosine transport protein) are unable to grow in the presence of low concentrations (3 μg/ml) of hypoxanthine.

DNA Preparation and Analysis. Plasmid DNA from *E. coli* was prepared according to Clewell (11). Yeast DNA preparation was carried out as described (12). Restriction endonuclease digestion, filling of protruding ends, and ligation of DNA were carried out as suggested by the suppliers.

Transformation. Yeast was transformed by the method of Hinnen *et al.* (13), except that Zymolase 60,000 at a final concentration of 50 μg/ml was used to prepare the spheroplasts. *E. coli* was transformed by the method of Cohen *et al.* (14).

Measurement of the Initial Rate of Uptake for Purine Bases. Initial uptake rates were measured as in ref. 6. Exponentially growing cells or stationary cells after 60 min of preincubation were incubated for 1 min with [^{14}C]hypoxanthine. The cells were separated from the reaction mixture by filtration over glass fiber filters. The filters were then assayed for radioactivity.

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Synthesis of 8-Azido[2-^{3}H]Adenine. Commercially available 8-azido[2-^{3}H]adenosine was used as starting material (250 μCi; 15 Ci/mmol in 2.5 ml of methanol; 1 Ci = 37 GBq). The methanol was evaporated under a gentle stream of nitrogen and the residue was then suspended in 0.5 ml of 1 M HCl and incubated for 15 min at 100°C. Thereafter, the sample was freeze-dried and dissolved in 2.2 ml of 0.01 M HCl. An almost 100% degradation into 8-azido[2-^{3}H]adenine was confirmed by TLC.

Photoaffinity Labeling. Cells were harvested in the early exponential phase of growth, washed once with distilled water, and then suspended in ice-cold distilled water (1×10^8 cells per ml). One milliliter of this cell suspension was pipetted into a glass dish (diameter, 5.5 cm), which was placed in an ice bath on a magnetic stirrer. 8-Azido[2-^{3}H]adenine was added to a final concentration of 0.5 μM. For testing the inhibitory effect of natural substrates of the purine-cytosine transport system on specific binding of the photoaffinity label, these substrates were added in thousandfold molar excess to the reaction mixture prior to irradiation. After 5 min of incubation, the cell suspension was frozen by pouring liquid nitrogen over it. The glass dish was then transferred to an ethanol/dry ice bath. The sample was irradiated for 5 min (1.9 mW/cm^2) with a UVS-54 Mineralight lamp (emission maximum, 254 nm). Thereafter the sample was quickly thawed and the cells were separated from the incubation mixture by filtration. The whole procedure was carried out in the dark.

Plasma Membrane Isolation. Plasma membranes were isolated and purified as described in ref. 8. The cells were converted into protoplasts and their plasma membranes were coated with cationic silica microbeads. After lysis, the plasma membranes were washed free of other cell organelles.

NaDodSO$_4$/PAGE. Electrophoresis was carried out as described by Laemmli (15). After photoaffinity labeling, the plasma membrane obtained from 4×10^8 cells was incubated for 5 min at 100°C in denaturation buffer [5% (wt/vol) NaDodSO$_4$/5% (vol/vol) 2-mercaptoethanol/10 mM Tris·HCl, pH 8]. After 5 min of centrifugation at 10,000 × *g*, the denaturated plasma membrane proteins in the supernatant were electrophoresed together with ^{14}C-labeled molecular weight standards using a 3-mm 8% slab gel.

After the run, the gel was cut into 2-mm strips, which were immediately placed to 10 ml of 3% (vol/vol) Protosol in Econofluor. The samples were incubated overnight at 30°C and then their radioactivity was assayed by liquid scintillation counting.

mRNA Analysis. RNA was extracted as described in ref. 16. Transfer of denatured RNA from agarose gels to nitrocellulose papers and hybridization to a [^{32}P]DNA probe labeled by nick-translation were carried out according to the procedure of Thomas (17).

Glycosidic Enzyme Treatment. Plasma membrane isolated from 8×10^8 cells after photoaffinity labeling was incubated for 2 min in 10% NaDodSO$_4$ at 100°C. After 5 min of centrifugation at 10,000 × *g*, the supernatant containing the extracted plasma membrane proteins was dialyzed for 2 hr against distilled water to remove the NaDodSO$_4$. Thereafter, the sample was freeze-dried. The resulting protein pellet was suspended in 1 ml of 50 mM sodium citrate buffer (pH 5.5) containing the following glycosidic enzymes: 0.1 unit of endoglycosidase H, 0.43 unit of neuraminidase, 8.5 units of α-mannosidase, 19 units of β-glucosidase, and 0.87 unit of galactosidase. After 3 hr of incubation at 34°C the mixture was dialyzed against distilled water, freeze-dried, and then suspended in 100 μl of denaturation buffer.

Tunicamycin Treatment. Cells were cultivated to a cell titer of 2×10^7 cells per ml and then 2.5 μg of tunicamycin per ml of growth medium was added. The cells were incubated for 2.5 hr to reach a final cell titer of 3×10^7 cells per ml.

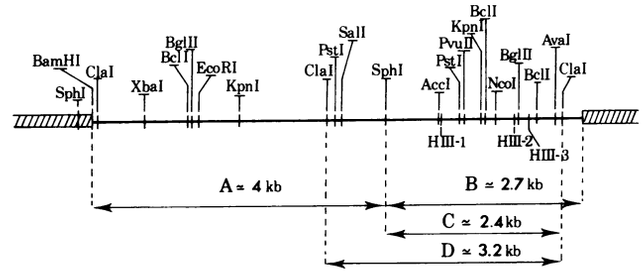

FIG. 1. Restriction map of yeast DNA insert into pNCII4-9. The horizontal bars and their corresponding letters indicate that these fragments were recloned into plasmid pJDB207 to test their complementation ability in yeast. Several *Hin*dIII sites found in fragment A are not shown on the map because their position was not determined.

Chemicals. 8-Azido[2-^{3}H]adenosine, the ^{14}C-labeled molecular weight standards, Protosol, and Econofluor were from New England Nuclear. ^{14}C-labeled purine bases and [^{14}C]cytosine were purchased from Amersham Buchler. Tunicamycin was from Sigma. The glycosidic enzymes were from Boehringer Mannheim except for endoglycosidase H, a product of Miles Laboratories. Restriction endonucleases were from Biolabs, Bethesda Research Laboratories, and Boehringer Mannheim. All other chemicals were of analytical grade or of the highest purity available. The lamp UVS-54 Mineralight was from Ultra Violet Products (San Gabriel, CA).

RESULTS

Cloning of the Purine-Cytosine Transport Protein Gene. Transformation of strain NC117spl with two independently

Table 1. Initial uptake rates for cytosine in transformed strains relative to wild type

Strain	Relative uptake rate
FL100	1
NC117spl (pJDB207)	0.013
Pool I	
NC117spl I 2	0.6
NC117spl I 3	2.5
NC117spl I 5	1.5
NC117spl I 6	2.7
NC117spl I 7	2.4
Pool II	
NC117spl II 1	2.4
NC117spl II 2	2.7
NC117spl II 3	2.2
NC117spl II 4	3.1
NC117spl II 5	1.5
NC117spl II 4–9	3.8
NC117spl pX	3.4
NC117spl pAB	3.4
NC117spl pZ	0.01

Strains with phenotypic complementation for purine-cytosine transport were selected and assayed for their transport activity relative to the wild-type strain FL100. NC117spl is a transformed transport-deficient mutant. The plasmids listed above are derivatives of plasmid II-4 with yeast DNA insert as shown in Fig. 1.

constructed yeast DNA pools yielded about 20 colonies that grew on hypoxanthine at 3 μg/ml. In 5 colonies derived from each pool, the initial uptake rates for cytosine were determined. These rates ranged from 0.6 to 3 times that of the wild type (Table 1). The DNA from clone II4 was extracted and used to transform *E. coli*. Plasmid DNA of ampicillin-resistant clones was analyzed. A plasmid with a 6.9-kilobase (kb) yeast DNA insert was found that restored purine-cytosine transport in uptake-deficient yeast strains.

A restriction map of the 6.9-kb fragment is shown in Fig. 1. Several fragments were subcloned in the vector pJDB207 and tested for their complementation ability in strain NC117spl. Fragments B and C (plasmids pX and pAB) with 2.9- and 2.6-kb inserts, respectively, complemented the uptake deficiency of the recipient strain whereas fragment A (plasmid pZ) did not (Table 1). Restoration of hypoxanthine uptake activity in the recipient strains could be the result of (*i*) introduction as a multicopy element of a transport protein distinct from the purine-cytosine transport system in which low affinity is balanced by high copy number, (*ii*) cloning of a suppressor gene, and (*iii*) cloning of the true purine-cytosine transport protein gene *FCY2*.

The first possibility is ruled out by the fact that strains containing the cloned gene exhibit the same substrate specificity and K_m values as the wild type (Table 2). Similarly, the increase in V_{max} by a factor of 3 above the wild-type level makes the cloning of a suppressor gene unlikely. To confirm this interpretation, the yeast strain NC203spl (*α ura3-373-251-328 fcy2-3*) was transformed by an integrating plasmid made of pBR322, the yeast gene *URA3*, and the yeast DNA fragment D, which contains the region complementing the purine-cytosine permease deficiency (Fig. 1). The use of a *ura3* recipient strain with three mutations scattered in the gene eliminated the recovery of prototrophic colonies resulting from integration at the *URA3* locus. Moreover, to enhance the integration frequency and to direct it to the chromosomal region homologous to fragment D, the plasmid was linearized (18) using the restriction enzyme *Bgl* II (Fig. 1). Three clones transformed to uracil prototrophy and purine-cytosine permease proficiency were then crossed with strain FL480-1D (*α fcy2-3*). Eleven tetrads were analyzed and gave a 2:2 segregation for the *fcy2-3* allele whereas eight uracil auxotrophs were recovered. This shows unambigously that the D fragment had integrated at the *FCY2* locus. Therefore, we conclude that the cloned DNA fragment contains the complete *FCY2* gene coding for the purine-cytosine transport system and that this gene is situated in the C region.

Localization of the Gene Within the Cloned DNA Fragment. To define the position of the gene within the C fragment of plasmid pAB, the *Hin*dIII sites of this segment were modified one by one by filling with complementary base pairs the 5′ protruding ends of DNA that had been partially digested with *Hin*dIII. The DNA was circularized with T4 DNA ligase and used to transform *E. coli* to ampicillin resistance. The plasmid structure of ampicillin-resistant colonies was determined. A *leu2 fcy2-18* yeast strain was then transformed to prototrophy with the modified plasmids and purine-cytosine

Table 2. K_m and V_{max} values for cytosine and hypoxanthine transport of wild-type strains and a strain carrying the cloned transport protein gene

	Hypoxanthine		Cytosine	
Strain	K_m, M × 10^6	V_{max}, amol/(cell no. × min)	K_m, M × 10^6	V_{max}, amol/(cell no. × min)
Wild type	4	10	2.8	15
II 4-9	4	31	3.0	27
RXII (diploid)	3	14	—	—

Table 3. Initial uptake rates for cytosine in strains carrying plasmids modified at the *Hin*dIII sites

Plasmid modification	Uptake rate, cpm/OD unit·min
pAB control, unmodified	96,700
pAQ35 filling at site 1	0
pAQ56 filling at site 2	2,160
pAQ55, deletion from site 2 to site 3	1,900
pAQ4 filling at site 3	120,380

Strain NC201sp2 (*a leu2-1 fcy2-18*) was used as recipient for the various plasmids. Initial rate of cytosine uptake was measured using 4 μM [^{14}C]cytosine with a specific radioactivity of 5 mCi/mol.

permease activity was scored by measuring both cytosine uptake velocity (Table 3) and resistance to 5-fluorocytosine. Destruction of the *Hin*dIII site 1 completely abolished the uptake of cytosine (plasmid pAQ35), giving resistance to 5-fluorocytosine similar to that of a strain with the *fcy2-18* mutation. On the other hand, modification of the *Hin*dIII site 3 (*pAQ4*) did not lead to any change relative to pAB. When the *Hin*dIII site 2 was destroyed (*pAQ56*) or when the short DNA fragment spanning from the *Hin*dIII site 2 to site 3 was deleted (*pAQ55*), cytosine uptake was significant but well below the level found for pAB or pAQ4. Also the resistance to 5-fluorocytosine was intermediate between those of the wild-type and *fcy2*-mutated strains. These results show that the *Hin*dIII site 1 is located within the *FCY2* gene whereas the *Hin*dIII site 3 is situated outside. As modifications at the *Hin*dIII site 2 reduce the uptake velocity by a factor of 50, this site is likely located near one end of the gene either in the coding region or in a region that modulates *FCY2* gene expression. Therefore, the DNA fragment that extends from the *Hin*dIII site 1 to the *Hin*dIII site 2 is a valuable probe for hybridization to the mRNA that codes for the purine-cytosine transport protein.

Photoaffinity Labeling. For the specific photoaffinity labeling of the transport protein, cells in the exponential phase of growth were irradiated in the presence of 0.5 μM 8-azido[2-^{3}H]adenine. To distinguish between specific and nonspecific binding of the photoaffinity label, other natural substrates for the purine-cytosine transport system were added (final concentration, 0.5 mM) to the reaction mixture prior to irradiation.

After photoaffinity labeling, the plasma membrane was purified and the proteins were extracted. For a better com-

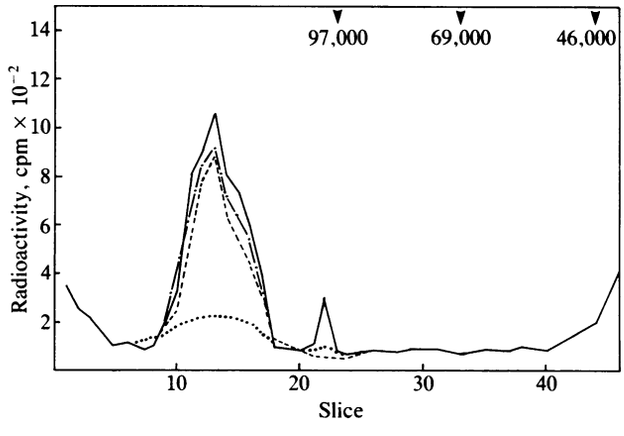

FIG. 2. NaDodSO$_4$/PAGE separation of plasma membrane proteins after photoaffinity labeling with 8-azido[2-^{3}H]adenine. Cells (4 × 10^8) were labeled in the absence (—) or presence of excess adenine (···), uracil (·—·), or ATP (---). Arrows indicate molecular weight markers.

parison, samples from different experiments were electrophoresed on the same gel (Fig. 2). Two major peaks of radioactivity were found, one with an apparent molecular weight of 120,000 and the other, of 100,000.

The specific labeling of the M_r 120,000 protein was largely blocked when adenine (or another natural substrate of the transport system) was added to the reaction mixture prior to irradiation (Fig. 2). In contrast, uracil or ATP, pyrimidine or purine derivatives that are not recognized by the transport system, did not interfere with photoaffinity labeling of the M_r 120,000 protein. ATP, however, blocked the labeling of the M_r 100,000 protein, indicating that this protein is not related to purine-cytosine transport. Further support for this conclusion was derived from another control experiment in which 8-azidoadenine was replaced by 8-azidoadenosine, which does not affect the uptake of purine bases or cytosine. In this case, the M_r 100,000 protein was labeled whereas the M_r 120,000 protein was not.

mRNA Analysis. The mRNA of the cloned gene was identified by hybridization of the *Hin*dIII site 1–site 2 gene fragment to a cellular RNA blot. As shown in Fig. 3, only one band, which represents the mRNA of the cloned gene, is visualized. Its size of 1.9 kb corresponds to a protein with a molecular weight of about 73,000.

To explain the different molecular weights for the purine-cytosine transport protein obtained by NaDodSO$_4$/PAGE and by mRNA analysis, we considered the possibility that the transport protein is a glycoprotein and that its carbohydrate moiety is responsible for the difference. A set of experiments was performed to check for this possibility.

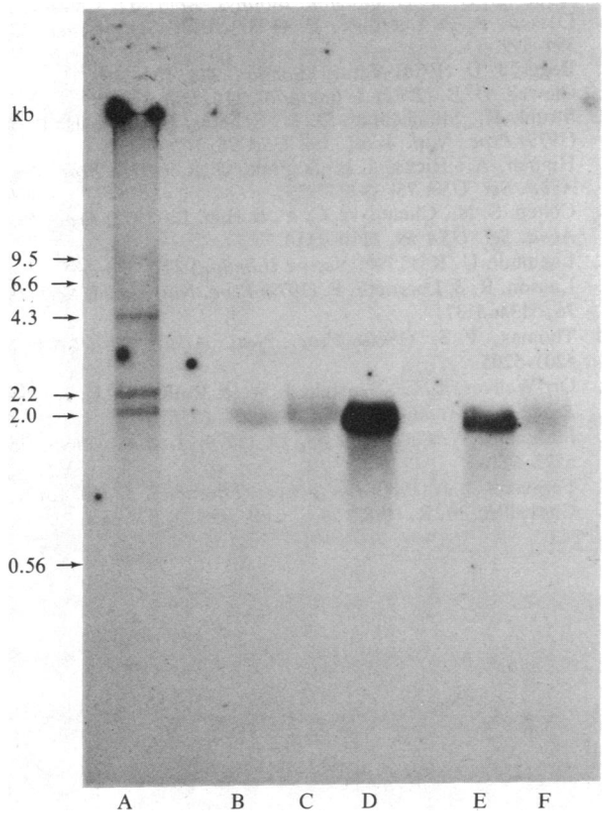

FIG. 3. mRNA blot. Wild-type yeast RNA was extracted, denatured, blotted to nitrocellulose paper, and hybridized to a ^{32}P-labeled DNA probe. The DNA probe was the 1-kb *Hin*dIII fragment (Fig. 1). Lanes: B and F, hybridization of the nonpolyadenylylated RNA fraction (100 μg per lane); D and E, hybridization of the polyadenylylated fraction (10 μg/ml); C, hybridization of total RNA (10 μg per lane); A, hybridization of reference phage DNA *Hin*dIII fragments.

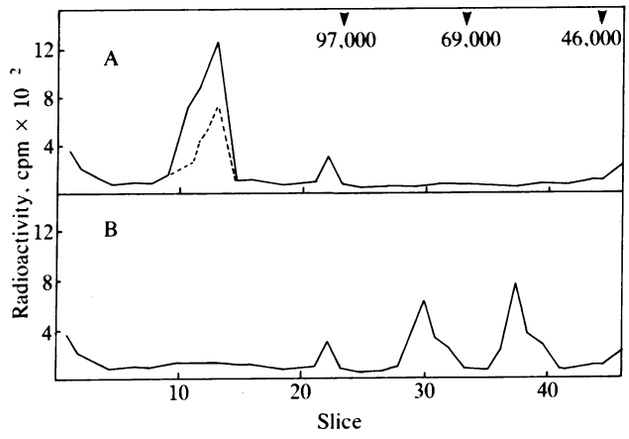

FIG. 4. Effect of tunicamycin treatment and treatment with glycosidic enzymes. (*A*) Cells were photoaffinity labeled with 8-azido[2-^{3}H]adenine before (—) and after (---) tunicamycin treatment. (*B*) Distribution of photoaffinity-labeled membrane proteins after treatment with glycosidic enzymes. Membrane proteins were separated by NaDodSO$_4$/PAGE on 8% gels.

Evidence for Glycosylation. In the first experiment, we used tunicamycin, an antibiotic that inhibits the transfer of *N*-acetylglucosamine-1-phosphate from UDP-(*N*-acetyl)-glucosamine to dolichol monophosphate and thereby blocks the formation of protein-carbohydrate linkage (19). The antibiotic was added to medium containing exponentially growing cells at a concentration of 2.5 μg/ml. During 2.5 hr of incubation, the cell titer increased from 2×10^7 to 3×10^7 cells per ml before cell division was blocked completely. Uptake experiments with [^{14}C]hypoxanthine showed that tunicamycin treatment caused a 40% reduction in the initial uptake rate.

The distribution of radioactivity after NaDodSO$_4$/PAGE of photoaffinity-labeled plasma membrane proteins extracted from the same number of cells before and after incubation with tunicamycin is shown in Fig. 4. The total amount of labeled M_r 120,000 protein after tunicamycin treatment was reduced by about the same extent as the initial uptake rate in the first experiment.

To separate the purine-cytosine transport protein from its carbohydrate moiety, we incubated the extracted plasma membrane proteins after photoaffinity labeling with a mixture of glycosidic enzymes. As shown in Fig. 4, after this treatment, NaDodSO$_4$/PAGE separation resulted in complete loss of the M_r 120,000 protein whereas the M_r 100,000 protein was unaffected and two previously unobserved labeled proteins appeared at M_r values of 60,000 and 73,000.

DISCUSSION

It has previously been shown that 8-azidoadenine binds covalently to the purine-cytosine transport system on UV irradiation (7). It was therefore tempting to use the radioactive compound for further characterization. Commercially available 8-azido[2-^{3}H]adenosine offers a suitable starting material for obtaining 8-azido[2-^{3}H]adenine by acid hydrolysis. We obtained high enough photoaffinity labeling to detect the protein after NaDodSO$_4$/PAGE by (*i*) using a mutant with the cloned transport system; (*ii*) labeling the cells in the frozen state, which increases the specific labeling (7, 20); and using a highly efficient procedure for purification of the plasma membrane [yield, ≈90% (8)].

The introduction of the cloned gene into a transport-deficient mutant not only restored its ability to transport but also increased the V_{max} to 3 times the level of wild-type strains without changing the K_m. This shows that we actually

cloned the purine-cytosine transport protein gene. Despite the fact that expression of the cloned gene was markedly less than level obtained with the cloned uracil transport protein gene (21), the use of a strain carrying the plasmid increased the specific labeling with 8-azido[2-^{3}H]adenine (results not shown). On the other hand, the cloned gene offered us a possibility to analyze transcription.

Two plasma membrane proteins are labeled by azidoadenine above the background level, one with a molecular weight of 120,000, the other of M_r 100,000. Only the M_r 120,000 protein can be protected against labeling by a variety of natural substrates: adenine, guanine, hypoxanthine, and cytosine (Fig. 2). Since no other protein is known to exhibit the same pattern of specificity, we conclude that the M_r 120,000 protein is actually the purine-cytosine transport protein. This conclusion is supported by the fact that uracil, which is closely related to cytosine but not a substrate for the transport system, does not inhibit the labeling (Fig. 2).

Labeling of the M_r 100,000 protein is prevented by adenine but not by other substrates of the transport system. ATP has the same effect (Fig. 2). Since nucleotides have no effect on the uptake of purine bases in *S. cerevisiae*, this protein is not related to the transport protein under investigation.

The discrepancy between the molecular weight of the transport protein as determined by $NaDodSO_4$/PAGE and as derived from mRNA analysis prompted us to investigate whether the labeled M_r 120,000 protein is a glycoprotein. We used tunicamycin, an inhibitor of protein glycosylation and glycosidic enzymes, to test this hypothesis.

The reduction of the overall uptake rate (Fig. 4) found after treatment of exponentially growing cells with tunicamycin could mean that daughter cells are depleted of a functional transport system. This possibility is supported by the finding that, in tunicamycin-treated cells, the total amount of the M_r 120,000 protein labeled by 8-azidoadenine is reduced to an extent that correlates with the decrease in uptake velocity (Fig. 4). Since no other labeled peak appeared, the M_r 120,000 protein seems to be a glycoprotein of which glycosylation and insertion into the plasma membrane is prevented by the antibiotic.

This hypothesis is further supported by the results obtained with glycosidic enzymes (Fig. 4). Treatment of photoaffinity-labeled membrane proteins with a mixture of glycosidases leads to the total disappearance of the M_r 120,000 peak but not of the M_r 100,000 peak and to the appearance of new peaks at M_r 60,000 and 73,000. Whereas the M_r 73,000 peak exactly matches the molecular weight deduced from mRNA analysis, the M_r 60,000 peak could be the result of proteolytic activities contaminating the glycosidic enzymes.

The finding that the transport protein is a glycoprotein could also account for the low expression of the cloned gene compared with the expression achieved with the cloned uracil transport protein gene (21). This hypothesis is further supported by the fact that the total amount of the specific mRNA in a strain containing the cloned purine-cytosine transport protein gene is 20-fold above the level found in wild-type strains (unpublished results). This differs markedly from the results obtained with the cloned uracil permease gene, where a commensurate increase in quantity of the specific mRNA and uracil transport activity was found (21).

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