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PROLINE TRANSPORT BY *PSEUDOMONAS AERUGINOSA*

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SUMMARY

Characteristics of the proline transport system were investigated in *Pseudomonas aeruginosa*. This transport system was found to obey saturation kinetics. From amino acid uptake and pool exchange studies the transport system was amino acid specific and specific for the L-isomer. This uptake system was also responsible for the entry of the proline analogues, thiazolidine-4-carboxylic acid, 3,4-dehydroproline, and azetidine-2-carboxylic acid. A mutant which specifically does not accumulate [^{14}C]-proline was isolated and characterized. Proline transport, the ability to degrade the amino acid, and the ability to concentrate proline were induced by growth in the presence of this amino acid. The ability to transport aliphatic or aromatic amino acids was reduced by growth in the presence of proline. The induction of the proline permease was reduced in the presence of glucose. Proline and not a degradation product of proline metabolism was found to be the inducer of this transport system. The accumulation of proline was shown to be N_3^- sensitive whereas the transport was unaffected.

INTRODUCTION

Escherichia coli has been shown to accumulate proline by an energy-dependent transport mechanism[1–3]. This transport system and especially the maintenance of intracellular pools has been recently critically reviewed[4]. The proline permease of *E. coli* was shown to be specific for this amino acid, however, KABACK AND STADTMAN[5], have found that proline accumulated in whole membrane preparations of *E. coli* and would still exchange with hydroxyproline. Otherwise, the accumulating system was amino acid specific. Recently, the specificity of the proline uptake system was again examined[6] and was found to be amino acid specific and responsible for the entry of a number of proline amino acid analogues.

This degree of specificity for proline uptake is not always maintained with other microorganisms, for instance BEHKI[7] found that the structural requirements for proline uptake in tumorogenic and non-tumorogenic strains of *Agrobacterium tumefaciens* were not as found for *E. coli*.

In a previous publication[8], we demonstrated that in *Pseudomonas aeruginosa* the maintenance of the intracellular proline pool differed from other amino acid pools in this organism. The formation of amino acid pools was mediated by an enzyme-like

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transport system, and the maintenance of preformed proline pools did not influence the formation of non-related amino acid pools, nor did any exogenous amino acid influence the level of the proline pool at steady-state. These results suggested that both the mechanism of the pool maintenance, and perhaps the proline transport system, were structurally specific.

In this report we investigated further the properties of the proline transport system in *P. aeruginosa* in glucose and proline grown cells and considered both the specificity and control of the uptake process.

MATERIALS AND METHODS

Bacterial strains

The microorganisms used in this study were *P. aeruginosa* ATCC 9027 and *P. aeruginosa* P22, a strain unable to degrade proline as a carbon or nitrogen source, and *P. aeruginosa* P5, a strain unable to accumulate proline.

Growth conditions, media, maintenance of stock cultures have been described previously[8,9].

Uptake of [^{14}C]proline

The general procedures for the measurement of [^{14}C]amino acid incorporation into whole cells and cell fractions of *P. aeruginosa* have been previously described[9]. The L-isomer of [^{14}C]proline was used in all transport experiments. In competition experiments for L-[^{14}C]proline uptake, [^{12}C]amino acids or analogues were added at 0.1 mM with 1 μM L-[^{14}C]proline to cell suspensions. Samples were filtered at 15-sec intervals for 1 min. The rate of amino acid incorporation was found to be linear over this time interval.

Studies on saturation kinetics of L-[^{14}C]proline uptake and the kinetics of competitive inhibition were carried out at 30° in reaction mixtures as previously described[9].

Pool exchange studies were performed at 10° using cells which were first pre-incubated at 30° with 200 μg/ml chloramphenicol. Pool exchange was initiated by the addition of the [^{12}C]amino acid or analogue to a final concentration of 0.1 mM.

Chromatography and radioautography of the intracellular pools was performed as previously described[9].

Growth studies

Growth studies were carried out at 37° on a temperature controlled Metabolite water bath (New Brunswick Scientific Co., New Brunswick, N.J.) using 250-ml erlenmeyer flasks equipped with 13-mm test tube side arms. Growth was followed turbidimetrically by measuring the absorbance of the culture at 650 nm.

Chemicals

L-[^{14}C]Proline was purchased from Schwartz Bioresearch Inc.; *N*-acetyl-DL-proline and chloramphenicol were obtained from Sigma Chemical Co. L-Thiazolidine-4-carboxylic acid was synthesized according to the procedure of RATNER AND CLARK[10], and SCHUBERT[11], as modified by MACKENZIE AND HARRIS[12]. 3,4-Dehydro-DL-proline was kindly supplied by Dr. B. Witkop, National Institutes of Health, Bethesda, Md. D-Proline, L-azetidine-2-carboxylic acid, D-azetidine-2-carboxylic acid, L-pipecolic

acid, and 4,5-dehydropipecolic acid (baikain) were purchased from Calbiochem. 2,4-Pyridine dicarboxylic acid, 2,5-pyridine dicarboxylic acid and 2,6-pyridine dicarboxylic acid were purchased from Aldrich Chemical Co.

RESULTS

The proline permease

In previous publications[8,9] we indicated that the proline uptake system of *P. aeruginosa* was temperature dependent and inhibited by uncouplers of energy metabolism, as was pool maintenance. We have also inferred that this transport activity was also subject to metabolic regulation[13]. Thus the proline uptake system had characteristics of other metabolic proteins. Fig. 1 demonstrates the adherence of the uptake process to Michaelis–Menten kinetics and confirms the enzymic nature of this amino acid transport system. The proline permease saturates at approx. 1 μM and as such has a relatively high affinity for the substrate proline. However, at substantially higher proline concentrations (Fig. 1 inset), a break in proline uptake was found to occur at approx. 20 μM. The second form of kinetics obtained in this manner indicated an apparent K_m of approx. 10-fold higher than that determined at low proline concentrations and a v_{max} value approx. 5-fold higher. It therefore appears that more than one parameter accounts for the permeability of *P. aeruginosa* to proline.

Specificity of the permease

The proline transport system was found to be strongly specific for this amino acid since the presence of 18 commonly occurring amino acids at 0.1 mM had no significant effect on the rate of [^{14}C]proline uptake into whole cells (Fig. 2). The majority of proline structural analogues previously tested for growth inhibition were found not

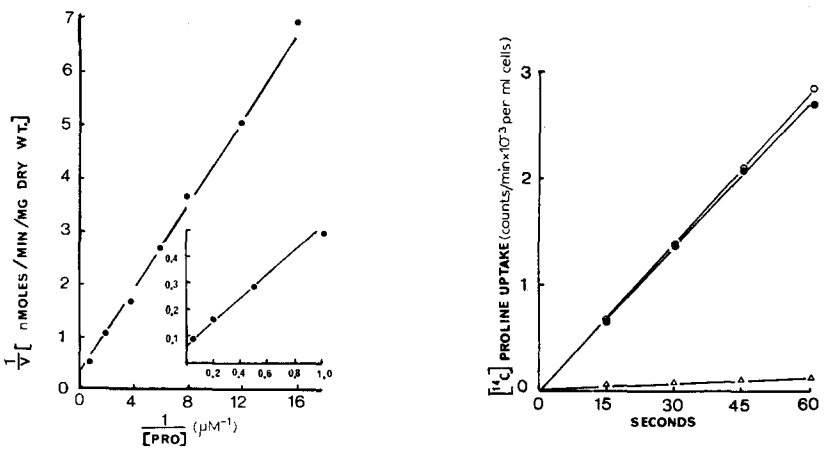

Fig. 1. Double reciprocal plot of [^{14}C]proline uptake by growing cells of *P. aeruginosa*. Inset: Double reciprocal plot at high proline concentrations. The cells (0.1 mg dry wt./ml) were incubated at 30° for 15 sec with labelled proline in a glucose minimal medium prior to harvesting millipore filtration.

Fig. 2. [^{14}C]Proline uptake by *P. aeruginosa* in the presence of 18 amino acids. The rate of [^{14}C]-proline (1μM) uptake (○–○) was determined in the presence of 0.1 mM [^{12}C]amino acids (●–●). [^{14}C]Proline uptake in the presence of 0.1 mM [^{12}C]proline was conducted as a control.

to be potent inhibitors of growth of this organism[13], suggesting that perhaps these analogues were in fact not permeable to the cell. When these analogues were tested as competitive inhibitors of the proline transport system it was found that only thioproline, dehydroproline, and L-azetidine-2-carboxylic acid inhibited the incorporation of [^{14}C]proline into *P. aeruginosa* to any detectable extent (Table I). The Lineweaver–Burk plot shown in Fig. 3 demonstrates the competitive inhibition of proline uptake

TABLE I

INHIBITION OF [^{14}C]PROLINE UPTAKE IN *P. aeruginosa* BY STRUCTURAL ANALOGUES

Uptake rates were determined from the initial (0–60 sec) incorporation of [^{14}C]proline (1 μM) into growing cells (0.1 mg dry wt. per ml) when added simultaneously with the [^{12}C]analogue (0.1 mM).

Competitor	*Rate of transport pmoles/min per mg dry wt.*	*Inhibition* (%)
None	580	0
L-[^{12}C]Proline	18	96.9
3,4-Dehydro-DL-proline	349	39.8
L-Thiazolidine-4-carboxylic acid	434	25.1
L-Azetidine-2-carboxylic acid	484	16.4
D-Proline	575	0.8
D-Azetidine-2-carboxylic acid	565	2.6
N-Acetyl-DL-proline	585	0
4-Nitropyridine-*N*-oxide	570	1.6
L-Pipecolic acid	580	0
4,5-Dehydropipecolic acid	581	0
Pyridine	572	1.5
2,4-Pyridine dicarboxylic acid	591	0
2,5-Pyridine dicarboxylic acid	570	1.6
2,6-Pyridine dicarboxylic acid	575	0.8

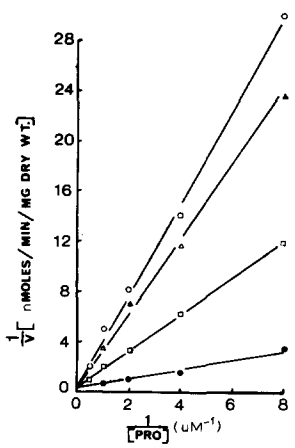

Fig. 3. Competitive inhibition of [^{14}C]proline uptake by amino acid analogues. 1 mM 3,4-dehydroproline (○—○); 1 mM thioproline (△—△); 1 mM azetidine-2-carboxylic acid (□—□); were added concomitantly with the labelled proline to cells which were vigorously stirred at 30°. Control without analogues (●—●).

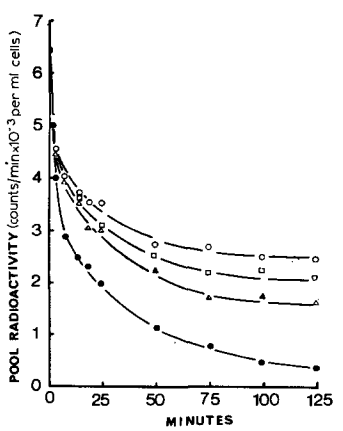

Fig. 4. Exchange of pool [^{14}C]proline at 10° with amino acid analogues. The proline pool was established from 1 μM [^{14}C]proline using 0.1 mg dry wt. per ml of cells pre-incubated with 200 μg/ml of chloramphenicol. Amino acid analogues added were: 1 mM azetidine-2-carboxylic acid (○—○); 1 mM thioproline (□—□); 1 mM dehydroproline (△—△) and 0.1 mM [^{12}C]proline (●—●) was added as the control.

by these analogues. Thioproline, dehydroproline, and azetidine-2-carboxylic acid were not only competitive for the uptake of [^{14}C]proline, but also were able to exchange with a pre-established [^{14}C]proline intracellular pool (Fig. 4). The ability to effect exchange by the analogue correlated directly with the relative degrees of inhibition of [^{14}C]proline uptake, thereby suggesting that the exchange of these analogues with proline was, in fact, a function of the activity of the proline permease. These three analogues effected [^{14}C]proline exchange in a manner similar to the addition of exogenous proline, that is, at a rapid rate.

Transport negative mutant

A mutant (P5), of *P. aeruginosa* was isolated as previously described[13] after mutagenesis with *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine. Growth characteristics of this strain were identical to those of the parent organism with the exception that this organism grew more slowly in minimal medium with proline as a sole source of carbon. This mutant was found to be unable to transport [^{14}C]proline when added at low concentration to the culture medium (Fig. 5). Control experiments indicated that the uptake of labelled glutamate, arginine, tyrosine, or isoleucine were unaffected by this lesion, thereby demonstrating that the mutation was in fact specific for the proline uptake system. Growth of this mutant in glucose minimal medium in the presence of 0.1% proline did not result in increased transport rates for this amino acid. This indicates that the high proline transport rate observed with cells grown in the presence of L-proline was due to induction of the transport system observed with cells grown in glucose minimal medium. Spontaneous revertants of *P. aeruginosa* P5 were selected on minimal medium with proline as a carbon source. Of five such revertants all had concomitantly regained both the ability to grow on proline as a sole carbon source and the ability to transport proline normally when compared to the parent strain.

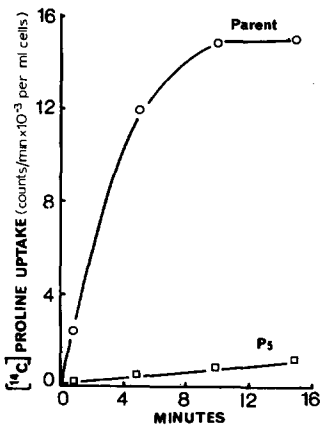

Fig. 5. The uptake of [^{14}C]proline 1 μM ,by wild-type (O—O), and the transport negative mutant P5 (□—□), of *P. aeruginosa* at 30°.

Induction of the proline permease

Preliminary experiments with cells grown in the presence of proline indicated that the ability to transport this amino acid was greatly increased. The time-course of proline pool formation in *P. aeruginosa* with cells incubated for a predetermined

interval with [^{12}C]proline is shown in Fig. 6. The simultaneous addition of chloramphenicol to the system completely prevented the induction of the proline permease. The kinetics of the induced proline transport system are shown in Fig. 7. At relatively low proline concentrations the apparent K_m values for both the induced and constitutive permease levels were identical within experimental error.

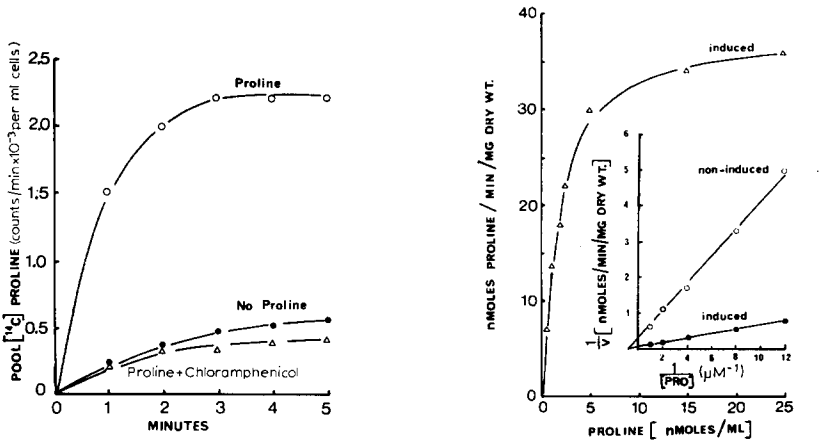

Fig. 6. The time-course of pool formation at 30° with *P. aeruginosa* grown in glucose minimal medium in the presence of 0.1 % proline (○—○), in the absence of added proline (●—●), or in the presence of proline and 200 μg/ml of chloramphenicol (△—△). [^{14}C]Proline was added at 1 μM to 0.2 mg dry wt. per ml of cells in the presence of 200 μg/ml chloramphenicol.

Fig. 7. Effect of proline concentration on the rate of [^{14}C]proline uptake by *P. aeruginosa* grown in the absence or presence of 0.1 % proline. Inset: Double reciprocal plot.

Effect of proline induction on other amino acid transport systems

Cells of the wild-type strain of *P. aeruginosa* grown in the presence of 0.1 % proline not only induced the proline permease but were found to also induce higher levels of the glutamate permease (Table II). However, the proline permease, but not the glutamate permease, was induced in *P. aeruginosa* P22, a mutant unable to utilise proline as a carbon source. This suggested that the induction of the glutamate permease in the

TABLE II

UPTAKE OF VARIOUS [^{14}C]AMINO ACIDS BY PROLINE INDUCED CELLS OF *P. aeruginosa*

Cells were grown on glucose minimal medium supplemented with 0.1 % L-proline. Uptake rates were calculated from the 0–60 sec incorporation of [^{14}C]amino acids into whole cells.

[^{14}C]*Amino acid*	*Uptake**	
	Parent	*P22*
L-Proline	690.6	608.6
L-Glutamate	470.6	96.2
L-Arginine	99.9	97.0
L-Tyrosine	77.8	75.2
L-Isoleucine	55.5	57.1
L-Hydroxyproline	0	–

*Amino acid uptake is expressed as a percent of the value obtained with glucose grown cells.

wild-type strain was in actuality a consequence of the degradation of proline to glutamate. The basic amino acid permease (unpublished results), represented here by the amino acid arginine, was unaffected, but unexpectedly, the ability of the cell to transport tyrosine or isoleucine was significantly reduced. The latter amino acids are representative of the aromatic and aliphatic transport systems respectively for this organism (unpublished results). Hydroxyproline was not transported detectably with these induced cells, nor was it transported by glucose grown cells[9].

When cells were grown in the presence of other amino acids, no significant effect was shown on the ability to transport proline (Table III). However, cells grown on proline alone as a sole carbon source were able to transport proline more effectively than cells grown in a glucose minimal medium with added proline. These results suggest that probably catabolite repression of amino acid permease occurs in this organism. The presence of glucose during the uptake experiment did not significantly affect the incorporation of [^{14}C]proline with proline grown cells.

TABLE III

THE UPTAKE OF [^{14}C]PROLINE BY *P. aeruginosa* GROWN IN THE PRESENCE OF VARIOUS AMINO ACIDS

Cells were grown in glucose minimal medium with the amino acid added at 0.1 %. Cells were harvested, washed twice and resuspended in fresh glucose minimal media immediately prior to the initiation of [^{14}C]proline uptake.

Amino acid added	[^{14}C]*Proline uptake**
None	1.0
Proline	5.2
Proline (in absence of glucose)	10.1
Glutamate, arginine, ornithine, isoleucine or tyrosine	1.0

* Uptake is expressed relative to the rate of [^{14}C]proline uptake with glucose grown cells.

Nature of the transport inducer

When wild-type cells of *P. aeruginosa* were grown in the presence of 0.1% proline, both the transport and the oxidation of proline were co-induced (Fig. 8). [^{14}C]Proline was rapidly incorporated then lost at a slower rate from the intracellular pool and was not recovered in the medium. This was not found to be the case with cells grown in glucose minimal medium[9]. However, mutants of *P. aeruginosa* which were unable to utilize proline as a sole carbon source could still be induced for proline transport (Fig. 9). Proline induced cells not only transported this amino acid more effectively than non-induced cells, but were also able to concentrate this amino acid approx. 10-fold over the non-induced cells. This intracellular pool was confirmed to be L-proline by chromatography and radioautography of the extracted pool.

Proline transport in N_3^--treated cells

It had been previously shown that high intracellular proline pools in *P. aeruginosa* were rapidly lost by exposure to uncouplers of energy metabolism such as NaN_3 (ref. 8). This demonstrated an energy requirement for the maintenance of high intracellular to extracellular concentration gradients. Since both the rate of transport and the ability to accumulate proline were increased by previous exposure to this amino

acid (Fig. 9), it was of importance to determine whether proline accumulation is an independent function or causually related to transport.

Cells of *P. aeruginosa* P22 with high transport activity (induced by growth on proline) were not able to accumulate [^{14}C]proline intracellularly when poisoned with N_3^- (Fig. 10). Increased amount of transport protein, therefore, does not facilitate the formation of concentration gradients over and above that previously observed with uninduced cells[8] even when both types of cells had been treated with N_3^-.

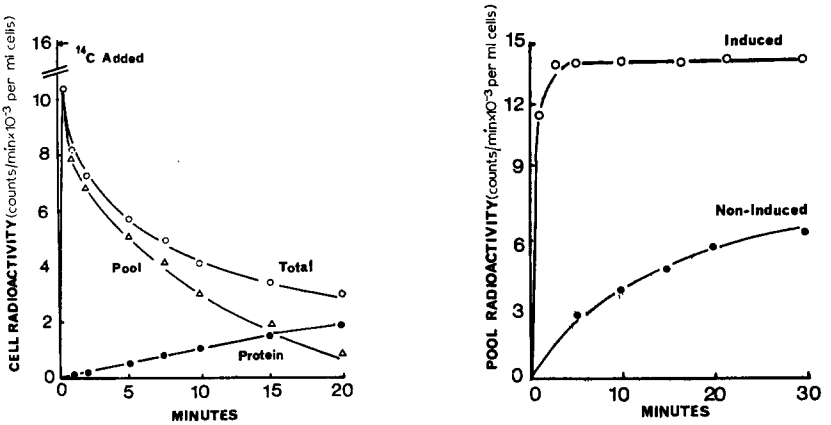

Fig. 8. The time-course of [^{14}C]proline uptake by *P. aeruginosa* grown in the presence of 0.1 % proline. [^{14}C]Proline (1 μM) was added to washed cells (0.1 mg dry wt. per ml) in glucose minimal medium. Pool radioactivity was determined subtractively.

Fig. 9. The incorporation of [^{14}C]proline (1 μM) into the pool of induced and non-induced cells of *P. aeruginosa* P22 at 10°. Induced cells were grown in glucose minimal medium with 0.1 % proline. Cells were pre-incubated at 30° for 30 min in the presence of 200 μg/ml of chloramphenicol prior to the addition of labelled amino acid.

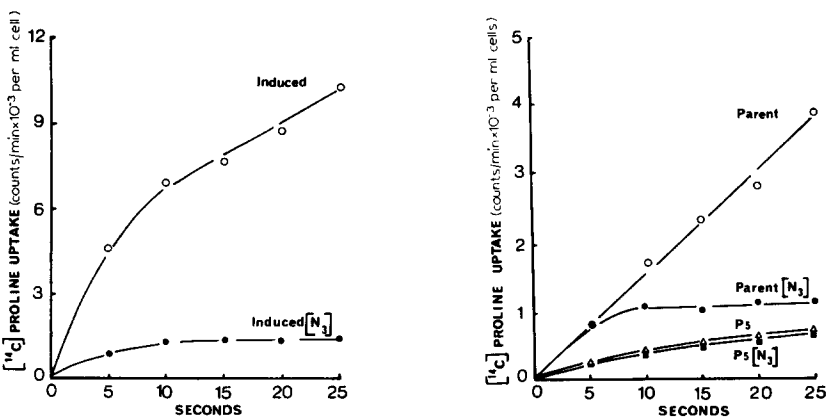

Fig. 10 Uptake of [^{14}C]proline (20 μM) into washed cells of *P. aeruginosa* poisoned with 30 mM NaN_3. Proline transport was induced by growth in glucose minimal medium with 0.1 % proline. Cells were pre-incubated at 30° with the inhibitor for 30 min prior to initiation of uptake.

Fig. 11. The uptake of [^{14}C]proline (20 μM) at 30° into NaN_3-treated and untreated cells of wild-type and the transport negative strain (P5) of *P. aeruginosa*. Cells were collected at 5-sec intervals by rapid Millipore filtration.

When the time-course of [^{14}C]proline uptake was followed with N_3^--treated cells (Fig. 11), it was observed that the initial rate of [^{14}C]proline uptake did not differ markedly from uninhibited cells and the intracellular proline pool was found not to exceed the extracellular proline concentration. These results suggested that not only were the N_3^--treated cells still permeable to proline, but also that the ability to transport proline was effectively unchanged. This treatment with N_3^- seemingly converted the uptake system from one of active transport to one of facilitated diffusion. As expected then, *P. aeruginosa* P5, the transport-negative mutant transported proline at a very low rate which remained unaffected by treatment with N_3^-.

From these results we observed that the initial rate of proline uptake could be measured with N_3^--poisoned cells. The kinetics of proline uptake determined with N_3^--treated cells (Fig. 12) demonstrated that the K_m and v_{max} values were not very different from uninhibited cells, and thereby indicated that the transport system *per se* was unaffected by N_3^- and that the accumulation function undoubtedly was.

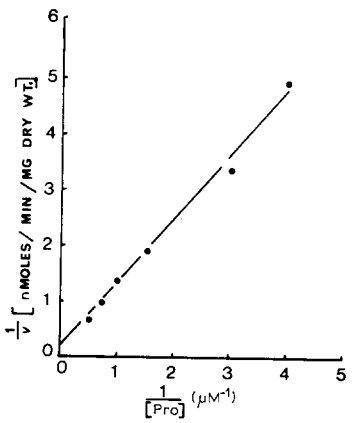

Fig. 12. Double reciprocal plot of [^{14}C]proline uptake by non-growing cells of *P. aeruginosa*, treated with 30 mM NaN_3 and 1 mM iodoacetamide. [^{14}C]Proline uptake rates were calculated from 5-sec incorporation data. K_m, 2.2 μM.

DISCUSSION

The uptake of proline by *P. aeruginosa* is energy and temperature dependent, can be lost by mutation, obeys saturation kinetics, and is apparently subject to regulation. These properties are commensurate with active transport mediated by an enzyme-like protein: a permease.

The kinetics of proline uptake show a break at high proline concentrations (20 μM). We have also found that non-linear double reciprocal plots are characteristic of many of the amino acid transport systems in this microorganism (unpublished data). These kinetics suggest that proline enters the cells by two distinct systems. The occurrence of two distinct apparent K_m values in amino acid transport systems have been previously described[14,16]. HALPERN AND EVEN-SHOSHAN[16] demonstrated non-linear double reciprocal kinetics for glutamate uptake with *E. coli*, and have proposed an allosteric permease model in which glutamate binds to an allosteric site and reduces the activity of the permease. Whether an allosteric or a second low affinity permease,

as described by AMES[14] explains the kinetics observed in this study, is unknown at present, nevertheless, at high amino acid concentrations the v_{max} of proline entry increases more than 5-fold, enabling the cell to incorporate enough proline to serve as both a carbon and energy source for growth in the absence of an alternate carbon source.

The high affinity proline permease was shown to be completely amino acid specific and also specific for the L-isomer of proline as has been demonstrated with *E. coli*[6]. Only the structural analogues thioproline, dehydroproline, and L-azetidine-2-carboxylic acid competed with proline for the permease, albeit with greatly decreased affinity relative to proline itself, indicating that the permease is very strongly stereospecific indeed. These analogues also were able to displace a preformed proline pool at 10°, and the degree of exchange correlated with the relative affinities for the proline permease deduced from the competitive inhibition of proline uptake. The limited exchange observed after approx. 50 min was probably due to the 'recapture' of exchanged proline by the proline permease. TRISTRAM AND NEALE[6] reported that L-azetidine-2-carboxylic acid, which has a low affinity for the *E. coli* permease, also exhibited limited displacement of a preformed proline pool. Under similar experimental conditions these three proline analogues were far less competitive than recently demonstrated for *E. coli*[6].

A non-specific proline transport system has been reported for *Agrobacterium tumefaciens*. BEHKI[7] has shown that a host of unrelated amino acids inhibited proline uptake in this microorganism.

The induction of permeases for carbohydrates has been reported[2]; however, there is little information on the induction of amino acid permeases. BOEZI AND DEMOSS[17] reported the induction of tryptophan transport in *E. coli*, but the system seems unusually complex since this induction required not only the amino acid inducer, but also the presence of cassamino acids. HALPERN AND EVEN-SHOSHAN[16] showed that growth of *E. coli* in the presence of glutamate caused a 40 % increase in the rate of glutamate uptake. LYON *et al.*[18] showed that induction of a glutamate transport system was requisite for the oxidation of glutamate in Mycobacterium species.

The proline permease of *P. aeruginosa* could be induced to much higher levels by growth in glucose minimal medium in the presence of proline. This induction required protein synthesis. Proline induced cells also induced the glutamate transport system, presumably due to increased pool levels of glutamate, a degradation product of proline metabolism. Unexpectedly, the activity of the aromatic and aliphatic amino acid transport systems were significantly reduced. INUI AND AKEDO[19] have demonstrated that the uptake of cycloleucine and leucine was reduced when cells were grown in a synthetic medium containing cycloleucine, leucine or methionine. It is possible, then, that some of the amino acid permeases are repressed in *P. aeruginosa* by growth in the presence of a structurally unrelated amino acid. The reason for this is at present unexplained. Cells grown in the presence of other amino acids transport proline at the same rate as cells grown in glucose minimal medium.

Cells grown on proline as a sole carbon source had higher proline transport activity than glucose–proline grown cells, suggesting that catabolite repression may be affecting the synthesis of the permease, however, no diauxic effect could be obtained in growth experiments with glucose and proline as sole carbon sources, in fact, *P. aeruginosa* grew faster with the combination of carbon sources than on glucose or proline

minimal medium. JACOBY[20] has shown that the degradation of 18 amino acids by *Pseudomonas fluorescens* could be prevented by the addition of glucose to the growth medium. Apparently, this mode of catabolite repression also affects the permeability of the respective amino acids. We have further shown that glucose, or perhaps an intermediate of glucose catabolism, strongly represses the synthesis of the aromatic amino acid permeases of *P. aeruginosa* (unpublished results). BOEZI AND DEMOSS[17] reported that glucose had no influence on the induction of the tryptophan transport system of *E. coli*.

In *P. aeruginosa*, both the transport of proline and proline catabolism were coordinately induced; however, each function could be mutated separately since Strain P22 was still inducible for proline transport but was unable to degrade the amino acid. It is possible that these functions are coordinately regulated. DE HAUWER *et al.*[21] have provided evidence that arginase, ornithine transaminase, and the arginine permease constitute an operon in *Bacillus subtilis*. In *P. aeruginosa* the inducer of the proline transport system would seem to be proline and not a degradation product thereof since strains which would not oxidize proline still were able to induce the transport system and proline was not found to be metabolized further.

NaN_3 apparently does not exert its inhibitory effect upon the rate of entry of the amino acid into the cells since initial rates and kinetics of proline uptake with N_3^--poisoned cells were similar to that observed for uninhibited cells. These results implicate a second energy dependent function in the maintenance of high intracellular pool levels. The maintenance of concentration gradients has physiological significance since most amino acids found in the intracellular pool of *P. aeruginosa* during growth on glucose are maintained at approximately a 200-fold concentration ratio[9].

The mechanism by which bacteria transport and maintain amino acid pools is by no means clear at present. No chemical change has yet been found to occur to the amino acid being transported[22]. We think that in *P. aeruginosa* it is likely that the formation and maintenance of the intracellular pool is a function of the particular permease. If the permease maintains the equilibrium between internal and external amino acids then an energy-dependent function must shift the equilibrium of the transport reaction in favor of pool formation. This is the view taken for β-galactosidase accumulation in *E. coli*[23]. HALPERN[24] has demonstrated differences in the entry and exit reactions for glutamate with *E. coli* and interprets this to mean that exit and entry mechanisms are in fact independent.

ACKNOWLEDGEMENT

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