RAPID PROLINE UPTAKE IN CULTURED TOBACCO CELLS AND INHIBITION BY A PROLINE ANALOG

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Received 30 March 1982

1. Introduction

Cultured plant cells present new opportunities for studies of metabolism and genetics [1–3]. Suspension cultures of tobacco cells offer an experimentally accessible system for investigating amino acid transport [4–10]. Cultured plant cells may be useful in elucidating underlying events associated with whole plant processes, such as studies of osmotic stress caused by drought or salt in higher plants [11,12]. Proline accumulation in whole plants and cultured cells that have been subjected to osmotic stress has been widely observed [13–17]. High intracellular proline concentrations may protect against water stress, perhaps by an osmolar effect [18,19]. Proline accumulation probably results from an increased rate of synthesis, though transport may be important in some cases [20]. Studies on proline transport have been reported for algae, plant tissues and organelles [21–25].

Cultured plant cells also offer the opportunity to select mutants and biochemical variants [26–28]. Amino acid analogs provide a specific type of selection pressure that may allow for the recognition of analog-resistant mutants in amino acid metabolism and transport. These mutants may be important in research and in practical applications [3,29]. The proline analogs L-azetidine-2-carboxylic acid and 4-hydroxy-L-proline have been used to select resistant mutants in cultured embryos [30] and cells [29,31,32]. Mutants in proline metabolism may be useful in determining whether proline accumulation confers some adaptive value to stressed plants or whether it is symptomatic of a deleterious response resulting from the stress.

Here we report our initial results on the rapid uptake of proline by tobacco cells in culture. Uptake is inhibited by the proline analog L-azetidine-2-carboxylic acid.

2. Materials and methods

2.1. *Growth and preincubation*

Cells of *Nicotiana tabacum* cv. Wisconsin 38, derived from callus culture, were grown in suspension in B5 medium as in [4]. B5 medium contains 1 mM Ca^{2+}. Exponentially growing cells taken 7 days or 14 days after routine subculturing were harvested, washed, and preincubated in 10^{-2} M bis–Tris–propane buffer (pH 5.5) containing 2% sucrose and 1 mM $CaCl_2$. Preincubation was at 23°C for 6 h unless otherwise indicated, with shaking at 120 rev./min in the dark and a cell density of 0.003–0.005 g/ml.

2.2. *Uptake*

After preincubation, L-[U-^{14}C]proline (New England Nuclear) was added at indicated concentrations, and incubation was continued for 20 or 30 min. The inhibitors such as L-azetidine-2-carboxylic acid, dinitrophenol, sodium azide, or carbonylcyanide *m*-chlorophenylhydrazone each filtering at 23°C and washing on the filter with a solution of nonradioactive L-proline at 10-times the concentration used during uptake. After excess liquid was removed by filtering, cell mats were weighed. Radioactivity was measured in Aquasol-containing scintillation fluid [4].

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Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

3. Results

The initial rate of proline uptake is very rapid, to an extent that the extracellular proline concentration began to decline after 20 min, even at 2×10^{-6} M proline. Under initial rate conditions, the uptake of proline was determined at various concentrations of [^{14}C]proline in calcium-containing buffer. Fig.1 is a double-reciprocal plot of the data obtained under 4 different conditions. Uptake is largely, if not entirely, mediated by a single process obeying Michaelis-Menten kinetics. Proline uptake is virtually the same for cells in mid-exponential and late-exponential phases (fig.1). With curve-fitting techniques using non-linear regression analysis of log-transformed velocity and substrate values, we estimated the parameters of uptake for a variety of simple binding and uptake equations. The analysis gives a good fit to an equation describing single carrier-mediated transport, with small terms for passive diffusion and adsorption. Under these conditions the biochemical parameters of uptake are $K_m = 1-2 \times 10^{-5}$ M and $V_{max} = 4000-5000$ nmol . g wet wt cells^{-1} . hour^{-1}.

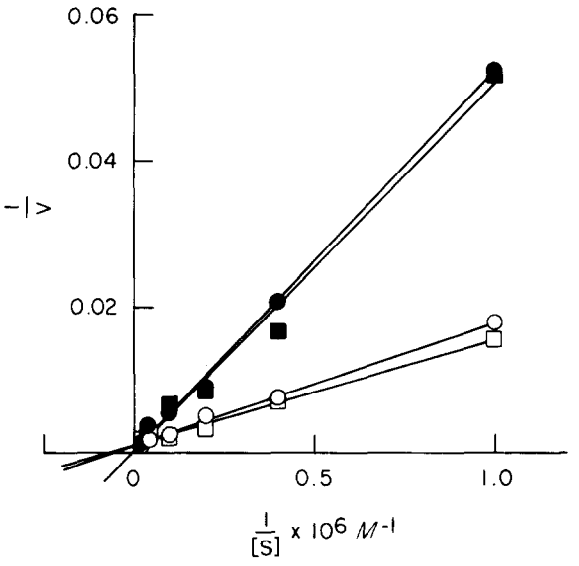

Fig.1. Double-reciprocal plot representing [^{14}C]proline uptake by *N. tabacum* cells in culture. Cultures were taken in mid-exponential phase, 7 days after transfer (□,■), or late-exponential phase, 14 days after transfer (○,●). The proline analog L-azetidine-2-carboxylic acid was present at 50 μM (●,■) or absent (○,□). Initial rates were measured as nmol [^{14}C]proline . g wet wt cells^{-1}. 20 min^{-1}. All the regression lines have correlation coefficients >0.98.

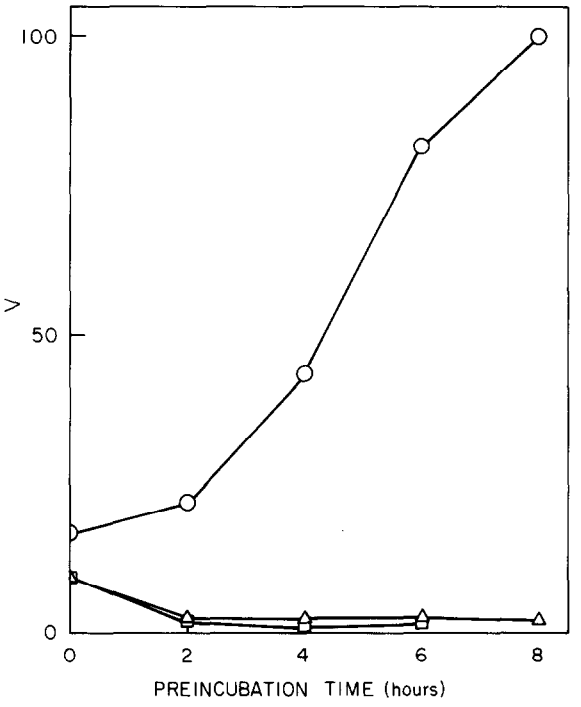

Fig.2. Dependence of [^{14}C]proline uptake on preincubation in the presence of Ca^{2+}. *N. tabacum* cells were harvested and washed in buffer without calcium, then incubated in suspension for the indicated time. Added [^{14}C]proline was 2×10^{-6} M, and uptake was measured for 20 min. Calcium was present during preincubation and labeling (○) at 1 mM, absent during preincubation and labeling (△), or absent during preincubation only (□).

Proline transport has an absolute requirement for calcium divalent cation during preincubation. Fig.2 shows that cells preincubated in sucrose buffer without calcium (or proline) do not later transport proline, whether or not calcium is present during the incubation with proline. The small amount of [^{14}C]-proline associated with the cells after 20 min incubation in proline is due to diffusion. Preincubation for 6–8 h in 1 mM $CaCl_2$ gives high rates of uptake, as reported for other amino acids transported by tobacco cells [4–7]. Added Ca^{2+} is not required during uptake itself (not shown) but must be present during preincubation.

Proline uptake in B5 growth medium (containing Ca^{2+}, no washes) is almost as effective as uptake in buffer after 6 h in preincubation, indicating that starvation or nutritional stress is not necessary for effective uptake. We investigated the role of calcium in

Table 1
Effects of inhibitors on uptake of proline by *N. tabacum* cells

Inhibitor	Conc. (M)	Rate of uptake[a]		Inhibition (%)
		Uninhibited	Inhibited	
Sodium azide	–	230.4, 222.3		
	1×10^{-4}		97.2, 101.1	56.2
Dinitrophenol	–	209.6, 221.8		
	2×10^{-4}		1.72, 1.82	99.2
	5×10^{-4}		0.30, 0.12	99.9
Carbonylcyanide *m*-Chlorophenyl-hydrazone	–	227.4, 222.6		
	1×10^{-6}		37.8, 29.4	85.1
	1×10^{-5}		0.87, 0.93	99.6

[a] Uptake measured during 20 or 30 min, rate calculated as nmol [^{14}C]proline . g fresh wt cells^{-1} . h^{-1}; [^{14}C]proline was 2×10^{-6} M in all cases

uptake by examining the proteins released by washing or synthesized during preincubation to identify calcium-specific membrane proteins. Thus far no particular protein has been implicated as specific for the calcium effect.

Inhibitors and uncouplers of oxidative phosphorylation (table 1) greatly inhibit proline uptake, suggesting that active transport is the major component of uptake.

Fig.1 also shows the inhibition of [^{14}C]proline uptake by Z-azetidine-2-carboxylic acid. Inhibition is competitive (or closely approximates simple competition). The K_i is $2–3 \times 10^{-5}$ M, similar to the K_m for proline. In addition, this proline analog is a very effective growth inhibitor in B5 medium, presumably because it is taken up by cells and incorporated into protein in place of proline [33–34]. Resulting proteins may be less active or inactive, slowing growth and causing lethality in cells. Proline added to B5 growth medium relieves the growth inhibition of L-azetidine-2-carboxylic acid. The $K_{0.5}$-value for proline's relief of inhibition by the analog is near the K_m for proline transport (not shown).

4. Discussion

These results indicate that tobacco cells in culture actively take up proline at a high rate. The high affinity and specificity of the proline transport system are shown by the relatively low half-saturation concentration and by the effective competition by L-azetidine-2-carboxylic acid. The proline analog is also a strong growth inhibitor of *N. tabacum* and other cells in culture.

Analogs of amino acids may allow for the selection of mutant or variant cell lines that are resistant to their inhibitory effects. An obvious type of resistance to an analog is due to failure to transport it under conditions where the natural amino acid is not required because of biosynthesis.

Analog-resistant mutants selected in cell culture may further help to elucidate endogenous metabolic pathways and provide varieties with altered amino acid contents. For instance, increased biosynthesis of an amino acid may confer resistance to its analog because the internal pool of free amino acids outcompetes the analog and prevents lethal protein synthesis. This kind of selection may be valuable for developing certain food plants with the desirable property of increased content of essential amino acids [3,29].

Proline uptake in *N. tabacum* cells in culture offers a promising experimental system for testing these possibilities. The following characteristics apply:

1. Proline is transported at a high rate even at low concentrations. Diffusion is a very small component of total uptake.
2. Proline transport is mediated by a single carrier, unlike lysine transport [4]. Multiple carriers make single-step transport mutants unlikely.
3. The proline analog L-azetidine-2-carboxylic acid effectively competes with proline for uptake and strongly inhibits growth. The lysine analog *S*-2-aminoethyl-L-cysteine is not nearly as effective at

inhibiting lysine uptake; high concentrations are needed and it is not a simple competitive inhibitor [4].

Should mutants or resistant variants arise in culture, *N. tabacum* plants may be regenerated. Analog-resistance in whole plant tissues is also testable [2].

Proline accumulation occurs in some plants under water stress, such as dehydration, freezing and salinity [13–17]. Resistance to water stress is of great economic importance. Experimental studies of the role of proline transport and biosynthesis in plant cells may apply to the development of agricultural and commercial plants that are now poorly adapted to growth under marginal conditions.

Acknowledgements

We thank Dr M. J. Constantin for providing laboratory facilities and helpful advice and encouragement. Research was supported by The University of Tennessee Institute of Agriculture and US Department of Energy Contract no. DE-AC05-760R00242 with The University of Tennessee. R. L. B. was a Summer Faculty Participant sponsored by the Department of Energy and Oak Ridge Associated Universities.

References

[1] Bottino, P. J. (1975) Rad. Bot. 15, 1–16.
[2] Widholm, J. M. (1979) Propagation of Higher Plants through Tissue Culture: A Bridge between Research and Application (Hughes, K. et al. eds) pp. 189–199, National Technical Information Service, US Department of Commerce, Springfield VA.
[3] Maliga, P. (1980) Int. Rev. Cyt. suppl. 11A, 225–250.
[4] Harrington, M. and Henke, R. R. (1981) Plant Physiol. 67, 373–378.
[5] Harrington, H. M., Berry, S. L. and Henke, R. R. (1981) Plant Physiol. 67, 379–384.
[6] Berry, S. L., Harrington, H. M., Bernstein, R. L. and Henke, R. R. (1982) Planta, in press.
[7] Smith, I. K. (1978) Plant Physiol. 62, 941–948.
[8] Berlin, J. and Mutert, U. (1978) Z. Naturforsch. 33c, 641–645.
[9] Harrington, H. M. and Smith, I. K. (1977) Plant Physiol. 60, 807–811.
[10] Blackman, M. S. and McDaniel, C. N. (1980) Plant Physiol. 66, 261–266.
[11] Nabors, M. W., Daniels, A., Nadolny, L. and Brown, C. (1975) Plant Sci. Lett. 4, 155–159.
[12] Nabors, M. W., Gibbs, S. E., Bernstein, C. S. and Meis, M. E. (1980) Z. Pflanzenphysiol. 97, 13–17.
[13] Stewart, C. R. and Hanson, A. D. (1980) Adaptation of Plants to Water and High Temperature Stress (Turner, N. C. and Karmer, P. J. eds) Wiley, New York.
[14] Greenway, H. and Munns, R. (1980) Annu. Rev. Plant Physiol. 31, 149–190.
[15] Boggess, S. F. and Stewart, C. R. (1980) Plant Sci. Lett. 17, 245–252.
[16] Stewart, C. R. (1980) Plant Physiol. 66, 230–233.
[17] Yelenosky, G. (1979) Plant Physiol. 64, 425–427.
[18] Csonka, L. N. (1980) in: Genetic Engineering of Osmoregulation (Rains, D. W. et al. eds) pp. 35–52, Plenum, New York.
[19] Jefferies, R. L. (1980) in: Genetic Engineering of Osmoregulation (Rains, D. W. et al. eds) pp. 135–154, Plenum, New York.
[20] Boggess, S. F., Stewart, C. R., Aspinall, D. and Paleg, L. G. (1976) Plant Physiol. 58, 398–401.
[21] McNamer, A. D. and Stewart, C. R. (1973) Plant Physiol. 52, 561–564.
[22] Tully, R. E., Hanson, A. D. and Nelson, C. E. (1979) Plant Physiol. 63, 518–523.
[23] Tully, R. E. and Hanson, A. D. (1979) Plant Physiol. 64, 460–466.
[24] Hanson, A. D. and Tully, R. E. (1979) Plant Physiol. 64, 467–471.
[25] Cavalieri, A. J. and Huang, A. H. C. (1980) Plant Physiol. 66, 588–591.
[26] Berlin, J. and Widholm, J. M. (1978) Z. Naturforsch. 33c, 634–640.
[27] Maliga, P. (1978) in: Frontiers of Plant Tissue Culture (Thorpe, T. A. ed) pp. 381–392, Int. Assoc. Plant Tissue Culture, Alberta.
[28] Henke, R. R. (1981) in: Propagation of Higher Plants through Tissue Culture: Emerging Technologies and Strategies (Constantin, M. J. et al. eds) Pergamon, Elmsford NY, in press.
[29] Widholm, J. M. (1977) in: Plant Tissue Culture and Its Bio-technological Applications (Barz, W. et al. eds) pp. 112–122, Springer-Verlag, Berlin, New York.
[30] Bright, S. W. J., Kueh, J., Franklin, J. and Miflin, B. J. (1982) Fourth International Barley Genetics Symposium, Edinburgh 1981, in press.
[31] Widholm, J. M. (1976) Can. J. Bot. 54, 1523–1529.
[32] Widholm, J. M. (1977) Crop Sci. 17, 597–600.
[33] Grant, M. M., Brown, A. S., Corwin, L. M., Troxler, R. F. and Franzblau, C. (1975) Biochim. Biophys. Acta 404, 180–187.
[34] Pitman, M. G., Wildes, R. A., Schaefer, N. and Wellfare, D. (1977) Plant Physiol. 60, 240–246.