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Regulation of Different Arginase Forms in *Evernia prunastri* Thallus

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Abstract

Arginase, or L-arginine amidinohydrolase, catalyzes the hydrolysis of L-arginine by producing ornithine and urea. Several oligomers of arginase are described, and are regulated by arginine analogues and lichen phenolics in *Evernia prunastri*. Atranorin as well as evernic and usnic acids modify the number of interaction sites of the enzyme with its own substrate (n_H), which, in absence of any effector, is always equal to 1.0. This fact indicates the feasible cooperativity in the binding of L-arginine or other molecules to arginase. The possibility of arginine movement between the different compartments of the thallus is discussed, as well.

Keywords: *Evernia prunastri*, arginine, arginine analogues, arginase oligomers, lichen phenols

Abbreviations: DTT, dithiothreitol; PVP, polyvinylpyrrolidone; Tris-HCl, tris (hydroxymethyl) aminomethane hydrochloride

1. Different forms of arginase in *Evernia prunastri*

Arginase, or L-arginine amidinohydrolase (E.C. 3.5.1.3.), is a hydrolysis-catalyzing enzyme by which L-arginine is converted to L-ornithine and urea (Fig. 1). Its occurrence in animal tissues (Skrzypel-Osieka et al., 1983; Beruter

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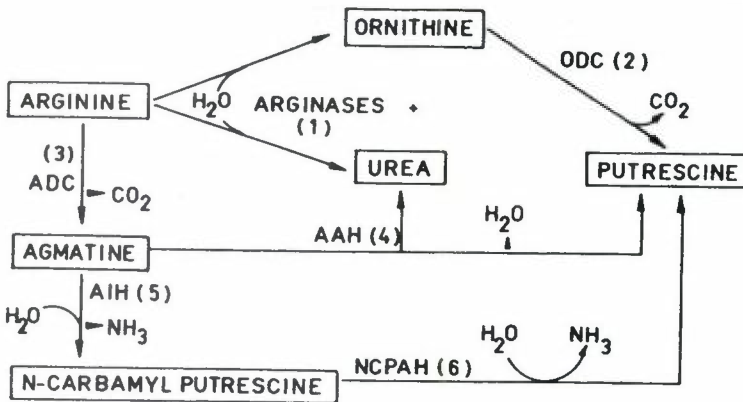

Figure 1. Metabolic pathways of L-arginine catabolism in *Evernia prunastri* thallus. Enzymes are (1), arginase; (2) ornithine decarboxylase; (3) arginine decarboxylase; (4) agmatine amidinohydrolase; (5) agmatine iminohydrolase and (6) N-carbamyl putrescine amidohydrolase.

et al., 1978; Spolarics and Bond, 1988) as well as in plants (Legaz and Vicente, 1982; Boulter and Barber, 1963; Ruiter and Kollofel, 1982; Splittstoesser, 1969) is widespread.

Maximum arginase activity can be detected in *Evernia prunastri* at different times, depending on the incubation media as well as on the light/dark conditions.

Arginase activities in the dark

The first arginase form is induced by its own substrate, L-arginine, since no activity is observed when cycloheximide, an inhibitor of translation, is included in arginine-containing media for the full time of incubation. The maximum enzyme activity is achieved at 6 hr incubation (Fig. 2) when thallus samples are floated on 40 mM L-arginine (Legaz and Vicente, 1980, 1982). In the following, this arginase form is designated **oligomer 1**.

The second arginase form is designated **oligomer 2** and is a pre-existent and inactive protein (Martin-Falquina and Legaz, 1984) which is activated by L-arginine, possibly mobilized from the vacuolar system to the cytosolic compartment, as happens in other organisms (Weiss and Davis, 1973). Its maximum activity is achieved at 16 hr incubation of the thalli in 40 μM cycloheximide in the lack of an exogenous supply of arginine (Fig. 2).

The third form of arginase (**oligomer 2**), which is induced by L-arginine as well, is secreted to the incubation media (Fig. 3). Since arginase is only secreted under conditions of nutritional induction, the secreted enzyme is presumably

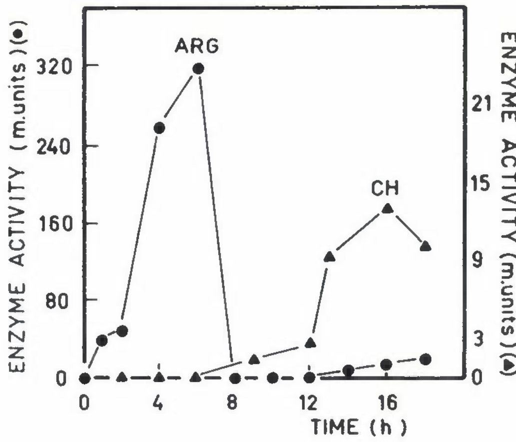

Figure 2. Time-course of arginase activity from *E. prunastri* thalli incubated in 40 mM L-arginine in 0.1 M Tris-HCl buffer (•) or in 40 μM cycloheximide in the same buffer (▲), in the dark, at 26°C. No activity was found when cycloheximide was added to arginine-containing media. Taken from Martin-Falquina and Legaz, 1984.

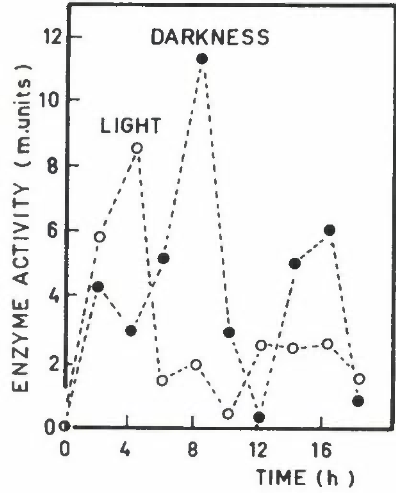

Figure 3. Time-course of arginase activity secreted to the media from *E. prunastri* thalli incubated in 40 mM L-arginine in 0.1 M Tris-HCl buffer in the dark (•) or in light (o) at 26°C. Taken from Planelles and Legaz, 1987.

the same induced protein as the one retained in the thallus. A polysaccharide of about 65 kDa is attached to this enzyme before or during secretion of the protein to the media (Planelles and Legaz, 1987). HPLC analysis revealed that this polysaccharide contained 280 molecules of glucose, 27 of fructose, and 85 of mannose per molecule of glycoprotein.

Arginase activities in light

In light, we can detect maximal arginase activities at 4 hr and 12 hr of thalli incubation in 40 mM L-arginine, so these are designed **oligomers 4** and **5**, respectively (Fig. 4). These activities are levelled off when cycloheximide is supplied to arginine-containing media. Furthermore, a maximum of activity of a pre-existent arginase, **oligomer 6**, is detected at 8 hr of thalli incubation in light in 40 μM cycloheximide in the absence of exogenous L-arginine (Legaz et al., 1990a). There is also secretion of arginase to the media at 4 hr of

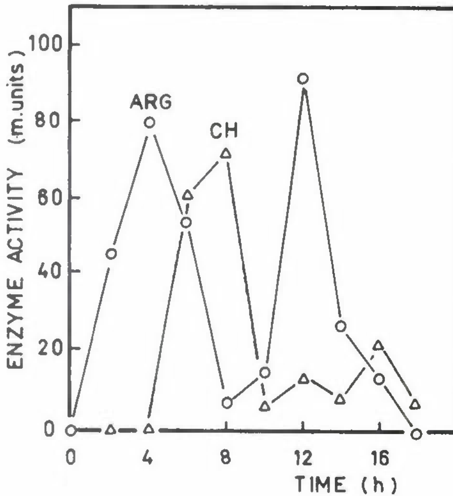

Figure 4. Time-course of arginase activity from *E. prunastri* thalli incubated in 40 mM L-arginine in 0.1 M Tris-HCl buffer (o) or in 40 μM L-arginine in 0.1 M Tris-HCl buffer (o) or in 40 μM cycloheximide in the same buffer (△) in light at 26°C. No activity was found when cycloheximide was added to argine-containing media. Taken from Legaz et al., 1990a.

thalli incubation in 40 mM L-arginine in light (Fig. 3). However, this form, **oligomer 7**, has not yet been purified and characterized.

The occurrence of different forms of arginase in the *E. prunastri* thallus raises the question of their physiological significance in the developing lichen thallus. Several observations indicate that arginine is synthesized *in situ* by lichen thalli. Although this amino acid is preferentially accumulated in the mycobiont of several lichen species (Solberg, 1970), the algal partner contains its own pool of L-arginine (Renner and Gerstner, 1982). The occurrence of a considerable amount of free L-arginine in the thallus of *E. prunastri* (Legaz et al., 1982) may indicate that it is not only incorporated into lichen proteins but also acts as a temporary N-storage compound. The annual variation in the amount of free L-arginine in *E. prunastri* thalli, as reported by Legaz et al. (1986), can be related to the seasonal induction of several forms of arginase, a process in which the amino acid acts as a nutritional inducer: the highest induced arginase activity corresponds to the highest amount of free L-arginine (inducer).

2. Some kinetical properties of the different forms of arginase

Arginase **oligomers 1** to **6** have been extracted and purified from thalli incubated when at their maximal activity. Table 1 summarizes some kinetic

Table 1. Some kinetic constants of different arginase oligomers

Oligomer of arginase	Incubation conditions for maximum activity	K_m (mM)	n_H	Mr calculated from sephadex G-200 (kDa)	Activation/inhibition constant
1 Thallus	6 hr 40 mM Arg in the dark	0.2	1	180	K_i = 2.6 mM (urea) K_i = 21.5 mM (agmatine) Evernic acid inhibits from 5.6 μM. Inhibition is complete from 16 μM
2 Thallus	16 hr 40 μM CH in the dark	2.5	1	330	K_i = 2.6 mM (urea) K_a = 2.7 mM putrescine K_a = 1.1 mM (ornithine) K_a = 5.8 mM (agmatine) Mixtures of lichen phenols activate it
3 Media	8 hr 40 mM Arg in the dark	1.5	1	245	K_a = 0.19 mM (usnic acid) K_a = 0.19 mM (usnic acid)
4 Thallus	4 hr 40 mM arg in light	2.1	1	330	K_a = 0.23 mM (evernic acid)
5 Thallus	12 hr 40 mM arg in light	1.92	1	264	K_a = 0.16 μM (atranorin)
6 Thallus	8 hr 40 μM CH in light	1.44	1	308	K_a = 33 μM (atranorin)
7 Media	4 hr 40 mM arg in light	?	?	?	?

CH = cycloheximide

properties of these arginase forms. All of them are michaelian enzymes since their n_H value is approximately equal to 1.0. The parameter n_H indicates the number of interaction sites with the substrate. K_m values are also shown in Table 1. **Oligomer 1**, with a K_m value of 0.2 mM for L-arginine, shows the highest affinity for its own substrate compared with the other arginase forms, the K_m of which are higher than 0.2 mM. Constitutive arginase developed in the dark (**oligomer 2**) shows the lowest affinity by L-arginine. In fact, **oligomer 2** is seasonally produced only when the activity or **oligomer 1** fails (Legaz et al., 1986). During these periods, the decrease in the amount of accumulated L-arginine is lower than that found when induced arginase (**oligomer 1**) is actively produced (Legaz et al., 1986). Arginase isozymes are often classified according to the value of the K_m constant. For this reason, the apparent K_m value is also determined and many different values have been reported (Beruter et al., 1978; Garganta and Bond, 1986; Simon and Stalon, 1976). However, no correction was made for substrate depletion or inhibition by products (Pace et al., 1980). Although not all the oligomers here described for arginase are isozymic forms, differences in amino acid composition have been found for both constitutive and induced oligomers. However, only quantitative differences have been found for distinct forms of induced oligomers (Pedrosa and Legaz, unpublished).

The molecular masses of the different oligomers have been calculated at first from filtration through Sephadex G-200. On this basis, it could be postulated that different forms of arginase could be interconvertible forms of only one structural subunit, as suggested by Martin-Falquina and Legaz (1984). A similar situation has been described for animal (Carvajal et al., 1982) and bacterial (Soru, 1983) arginases.

Recent works indicate the heterogeneity in size and charge of arginase subunits, demonstrating that this enzyme may exhibit more than one pattern of subunit aggregation. The subunits of hepatic arginase have a molecular mass of 35–38 kDa. The enzyme behaves as an oligomer composed of three or four of these subunits (Spolarics and Bond, 1988). On the other hand, native arginase extracted and purified from *Neurospora crassa* migrates as a hexamer during gel-filtration chromatography on TSK-G3000 SW column, with an apparent molecular mass of 266 kDa and a subunit molecular mass of 38.5 kDa (Borkovich and Weiss, 1987). There are precedents for hexameric arginases. Arginase from *Bacillus licheniformis* is composed of subunits with a molecular mass of 33 kDa (Simon and Stalon, 1976). *Bacillus subtilis* (Issaly and Issaly, 1974) and *Iris hollandica* (Boutin, 1982) have also hexameric forms of arginase.

3. Regulation of the different forms of arginase

Metabolic analogues of L-arginine, substrate of arginase, as well as lichen phenolics, are able to regulate arginase activity of different oligomers.

Regulation by substrate analogues

Table 1 shows the activation/inhibition constants of *E. prunastri* arginase forms (Vicente and Legaz, 1983). Based on these results, we could speculate about the regulation of arginase oligomers produced in the dark. When *E. prunastri* thalli were incubated in the dark, we could detect two forms of arginase retained in the thallus as well as a secretable form. **Oligomer 1** was induced by L-arginine and was negatively effected by agmatine and urea. **Oligomer 2** is a constitutive, inactive protein that is activated by putrescine, ornithine, and agmatine, but also inhibited by urea, as is the induced arginase. Both forms are slightly inhibited by an excess of substrate, L-arginine.

Agmatine, a precursor of polyamine biosynthesis, is the essential metabolite able to regulate both induced and constitutive arginases, since it inhibits and activates, respectively, these proteins. Then, it seems to be that the production of polyamines is mediated, preferentially by the action of arginine decarboxylase and not by the sequenced actions of both arginase and ornithine decarboxylase.

Arginase produces ornithine and urea from arginine and just when urea is produced, it inhibits the induced form of arginase. Once putrescine has been synthesized, *via* arginine decarboxylase, the production of ornithine and urea can be performed *via* constitutive arginase. In the absence of polyamines, the production of ornithine and urea is preferably mediated by induced arginase.

At low concentrations of substrate (L-arginine), the hydrolysis of the amino acid is carried out, preferentially, by means of an induced arginase (Fig. 5), since its K_m value, 0.2 mM, is twelve times lower than that of the constitutive arginase ($K_m = 2.5$ mM). Under these conditions, urea is produced and then able to inhibit induced arginase (**oligomer 1**). Endogenous, and probably organelle-sequestered arginine can move to the cytosol and, there, activate constitutive and inactive arginase (**oligomer 2**). When constitutive arginase is active, urea is produced again and is able to inhibit constitutive arginase. Then, the excess of arginine is used to produce agmatine and putrescine. The latter molecule activates constitutive arginase while inhibiting the inducible one.

It must be concluded that urea is formed by the action of induced arginase on low concentrations of arginine. Putrescine is produced at high concentrations of L-arginine and this compound is able to activate constitutive arginase.

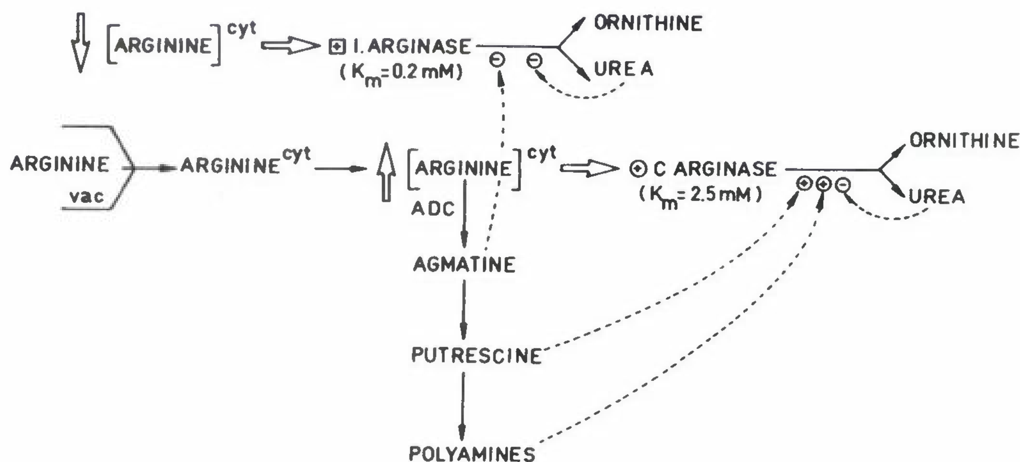

Figure 5. Hydrolysis of L-arginine by induced (**oligomer 1**) or constitutive (**oligomer 2**) arginase activity as well as decarboxylation of the amino acid by an arginine decarboxylase to produce polyamines. Cyt = cytosol; vac = vacuole; ADC = arginine decarboxylase.

Regulation by E. prunastri *phenolics (atranorin, and evernic and usnic acids)*

The different forms of arginase are also regulated by lichen phenolics. Table 1 shows the effects of some *E. prunastri* phenols (atranorin, and evernic and usnic acids) on arginase activities, as well as the values of activation/inhibition constants, at least for the oligomers produced in light and for the secreted protein detected in the dark. It is necessary to specify that these constants have been calculated on the basis of a specific concentration of lichen phenols. First of all, lichen phenolics dissolved in cell-free extracts, showing maximum specific arginase activity, were identified and their concentration measured by high performance liquid chromatography. Based on the results obtained above, these phenolics, at around the specific measured concentration for each one, were included in reaction mixtures containing purified arginase, in order to show the feasible changes in the Michaelian kinetic under these conditions. Since these concentrations mimick those naturally occurring in lichen cells (Avalos and Vicente, 1987), in the following, each phenol concentration dissolved in cell-free extract is designated **physiological effector concentration**. Table 2 shows **physiological effector concentration** of each phenol under the conditions of maximum arginase activity.

Oligomer 1 is strongly activated by chloroatranorin at a range of concentrations varying from 1 to 8 μM, but it is completely inactivated by concentrations

Table 2. Hill's equations for different arginase oligomers in the presence or in the absence of different effectors

Oligomer	Incubation conditions for maximum activity	Effector concentration (μM)	Equation in the absence of effector*	Equation in the presence of effector*
1	6 hr on arg in the dark	Evernic acid 12 μM	$y = 1.07x + 0.052; r = 0.98$	$y = 3.06x - 0.287; r = 0.95$
2	16 hr on CH in the dark	Evernic acid 2 nM	$y = 0.19x - 0.30; r = 0.93$	$y = 3.67x - 1.62; r = 0.99$
3**	8 hr on arg in the dark	Usnic acid 0.12 mM	$y = 0.87x - 0.14; r = 0.84$	$y = 1.22x - 0.34; r = 0.97$
4	4 hr on arg in light	Evernic acid 0.126 mM	$y + 0.81x - 0.29; r = 0.98$	$y = 1.69x - 0.89; r = 0.94$
5	12 hr on arg in light	Atranorin	$y = 0.91x - 0.25; r = 0.99$	$y = 0.55x - 0.11; r = 0.80$
6	8 hr on CH in light	Atranorin	$y = 0.93x - 0.21; r = 0.89$	$y = 1.31x - 0.38; r = 0.90$

* $y = \log(v_0/v_{max} - v_0)$; x is arginine concentration in the reaction mixture, as mM; n_H is the slope of the straight line

**Secreted enzyme

higher than 12 μM. Evernic acid totally inactivates the enzyme at concentrations higher than 16 μM. Inactivation can be partially reversed by including DTT in the incubation mixtures (Legaz and Vicente, 1983).

However, constitutive arginase (**oligomer 2**) is activated by lichen phenolics at the concentration found in buffered extracts which contain this enzyme activity. A mixture of 1.6 nM evernic acid, 1.4 nM usnic acid, and 1.46 nM atranorin activates arginase 1.6 times (Martin-Falquina and Legaz, 1984). Although these phenolics have been mainly described as inactivators of several enzymes (Vicente and Legaz, 1988), sometimes phenolics behave as activators of others. This phenomenon has been reported for laccase activity of *Agaricus bisporus* and *Pleurotus ostreatus* (Giovannozzi-Sernani and Luna, 1981). This is also the case of secreted arginase (**oligomer 3**), on which usnic acid acts as a non-essential activator of the enzyme with K_m value of 0.19 mM (Planelles and Legaz, 1987).

Atranorin and evernic acid are lichen phenols preferentially retained in the medula in *E. prunastri* thalli floated on L-arginine or on cycloheximide in the light (Legaz et al., 1990b). Induced arginase detected in light (**oligomer 4**) is activated by evernic acid, which is the only phenol that appears in buffered solutions in which thalli have been incubated for 4 hr. Atranorin is an activator of both **oligomer 5** and **6**, with K_m values of 0.16 μM and 33 μM, respectively. Thus, it is clear that evernic acid and atranorin may regulate induced arginases in light whereas the constitutive protein is regulated by atranorin.

Variation of n_H value in the presence of the effector

Arginase reaction rate *versus* different arginine concentrations varies in the presence or absence of the corresponding phenolic effector, as shown in Table 2, i.e. the phenol is able to change the number of binding sites for the substrate (n_H) in the arginase molecule. When n_H, the slope in the straight line of log [arginine] *versus* log $[v_0/v_{max}-v_0]$, is increased, positive cooperativity is meant. Positive cooperativity is said to occur when the binding of one molecule of a substrate (or ligand) to the enzyme increases the affinity of the protein for other molecules of the same or different substrate (or ligand). This fact occurs for all the **oligomers**, except for **oligomer 5**, where atranorin changes the n_H value from 1.0 to 0.55, displaying negative cooperativity (Legaz et al., 1990b). A similar situation has been described by Garganta and Bond (1986) for the inactivated rat liver arginase. That is, the presence of the activator would suggest a conformational change of the protein that increases or decreases the affinity of the enzyme by its own substrate.

It should be pointed out that apparent negative cooperativity, as exhibited for atranorin interaction with **oligomer 5**, would help to prevent accumulation of ornithine and/or urea by lichen thalli.

4. Regulation of the Different Forms of Arginase by Light/Darkness

As shown in Fig. 6, the extracellular pH value decreases in light, indicating that it increases inside the thallus. In the dark, pH value is maintained. Similar behaviour can be observed when lichen thalli are previously treated with PVP (which eliminates phenols), although the last pH decrease after 10 min of lighting is more marked. These results indicate that endogenous pH value increases in light whereas decreases, or it is maintained, in the dark.

There are many things about the molecular distribution between symbionts we do not yet know. We can not yet affirm whether arginine is inside the vacuole or other organelles, although, to some extent, previous data from other organisms, such as *N. crassa*, show arginine as a vacuolar metabolite (Weiss and Davis, 1973). Even in *E. prunastri*, the activation of **oligomer 2** in the absence of exogenous L-arginine at pH 6.5 after 16 hr of thalli rehydrated in buffered cycloheximide (Fig. 2) suggests that endogenously-produced amino acid moves in the mycobiont from one compartment to another. We have demonstrated (Martin-Falquina and Legaz, 1984; Legaz et al., 1986) that **oligomers 1** and **2** are not active at the same time and, therefore, cytosolic arginine is rapidly hydrolyzed to ornithine by means of induced arginase. After this hydrolysis,

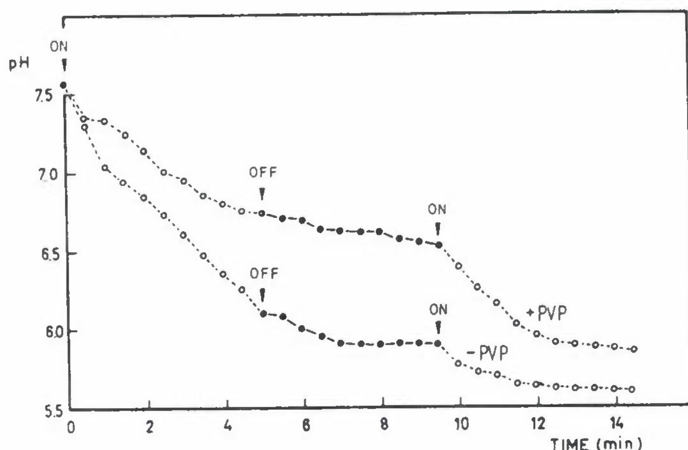

Figure 6. Time-course of exocellular pH produced by *E. prunastri* thallus incubated in distilled water (-PVP) or in 2% (w/v) PVP in distilled water in the dark (•) or in light (o).

the low concentration of cytosolic arginine is not enough to activate the constitutive arginase. Moreover, the K_m value of this last protein, which is twelve times higher than that of the induced arginase, indicates that the hydrolysis of arginine by means of **oligomer 2** needs a much higher concentration of cytosolic arginine than that which is available under natural conditions. Under these circumstances, hydrolysis of the amino acid that, in fact, is produced, is a consequence of arginine mobilization from the vacuolar system to the cytosol. This needs to be confirmed with histochemical and immunological analyses to locate both arginase and arginine molecules.

Figure 7 shows a possible scheme of arginine hydrolysis in both thalli and media, as well as the regulation of arginase forms by substrate analogues and *Evernia* phenolics.

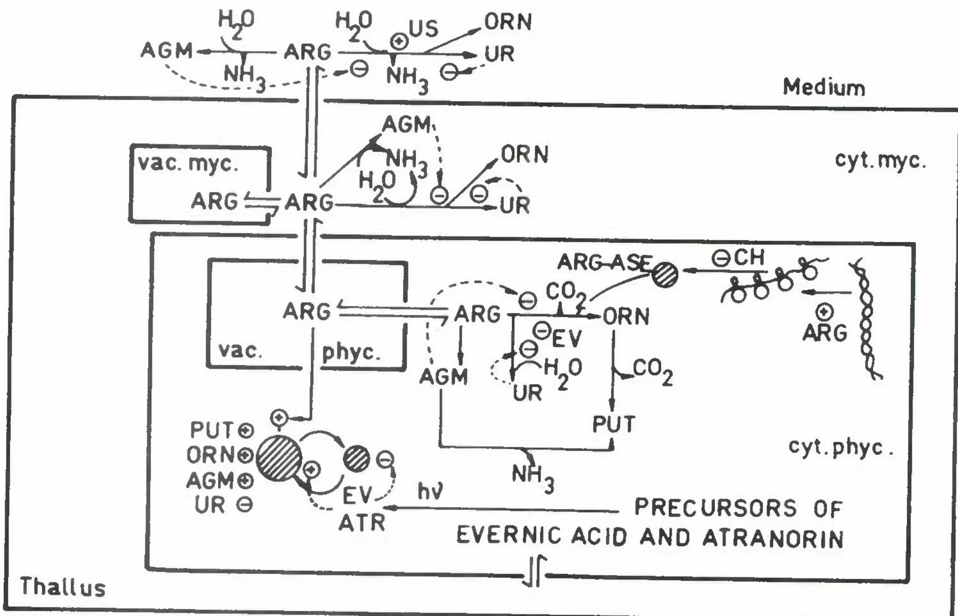

Figure 7. Scheme of the possible movement of L-arginine between cytosol, vacuoles and medium from *E. prunastri* thalli as well as the regulation of arginase oligomers by arginine analogues and lichen phenolics (broken lines). Abbreviations are: agm, agmatine; arg, arginine; ur, urea; orn, ornithine; put, putrescine; ev, evernic acid; us, usnic acid; atr, atranorin; ch, cycloheximide; arg-ase, arginase; vac myc, vacuole of the mycobiont; vac phyc, vacuole of the phycobiont; cyt cmyc, cytosol of the mycobiont; cyt phyc, cytosol of the phycobiont.

Mobilization of arginine in light

Light increases the endogenous pH value in lichen thalli. At alkaline pH values, **oligomer 1** is active whereas **oligomer 2** is not (Legaz and Vicente, 1982). Thus, urea is produced and inhibits this reaction. Arginine is then decarboxylated to produce agmatine, which also inhibits induced arginase. So, agmatine can be converted into putrescine. The increase in putrescine concentration might then activate **oligomer 2** (constitutive protein). Moreover, light increases evernic acid and atranorin production that also activate this form of arginase. In conclusion, the "short-term" hydrolysis of arginine is carried out by **oligomer 1** whereas a "long-term" hydrolysis is performed by the constitutive, **oligomer 2**.

Mobilization of arginine in the dark

In the dark, the endogenous pH value is lower than that found in the light. Under these conditions, **oligomer 2** is active whereas **oligomer 1** is not. Thus, hydrolysis of arginine is carried out by constitutive arginase (**oligomer 2**). Decarboxylation of arginine to produce agmatine also implies an activation of **oligomer 2**. This could be the "short-term" response. However, as a consequence of arginine mobilization by hydrolysis or decarboxylation, the decrease of cytosolic concentration of this amino acid would imply an inactivation of **oligomer 2** and, then, activation of induced arginase as a "long-term" response. Moreover, phenolic concentration in the dark is low and not enough to inactivate induced protein.

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