Binding Studies of Evernic Acid to Purified Constitutive Arginase of *Evernia Prunastri* by SE-HPLC

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Abstract

A constitutive arginase has been purified about 260-fold from *Evernia prunastri* thalli incubated for 16 hr on 40 μM cycloheximide. This enzyme has a molecular mass of 36.5 kDa and only one active site, which implies an absence of cooperativity when substrate binds to the enzyme. When evernic acid binds to arginase, the enzyme polymerizes and the number of interaction sites with the substrate increases from 1 to 3–4. This is interpreted as a mechanism of positive cooperativity in the binding of ligand to arginase.

1. Introduction

Arginase purified from several sources seem to be polymeric enzymes. Arginase from *Neurospora crassa* has a subunit molecular mass of 38.3 kDa, as

determined by SDS-PAGE (Borkovich and Weiss, 1987a), although the polymer can be dissociated in two major immunoreactive proteins of molecular mass 41.7 kDa and 36.1 KDa, identified with polyclonal antibodies (Borkovich and Weiss, 1987b). The native, polymeric enzyme from *N. crassa* behaves as a hexamer with an apparent molecular mass of 266 KDa; on the other hand, mouse liver arginase is an oligomer composed of four subunits with molecular weights of 35 kDa and 38 kDa, respectively (Spolarics and Bond, 1988).

Evernia arginase is a pool of different native proteins. Both induced and constitutive arginases have been characterized from *E. prunastri* thalli. The former has a molecular mass of 180 kDa (Legaz and Vicente, 1982), whereas the constitutive enzyme has a molecular mass of about 330 kDa (Martin-Falquina and Legaz, 1984). These molecular masses were estimated by gel filtration through Sephadex G-200. In spite of their possible different genetic origin, interconversions between induced forms of different molecular mass, effected by phenols, have been described (Legaz et al., 1990a) in a similar way to that reported by Aguirre and Kashe (1983) for oligomeric rat liver arginase.

This paper describes the follow up by SE-HPLC of the purification procedure of a constitutive arginase produced by *E. prunastri* thalli under the same experimental conditions as those described by Martin-Falquina and Legaz (1984), as well as the changes in the molecular mass of this protein effected by a natural activator of the enzyme, the depside evernic acid.

2. Materials and Methods

Plant material and incubation conditions

Evernia prunastri (.) Ach., growing on *Quercus pyrenaica* Lam., and collected in Valsain (Segovia, Spain), was used throughout this work. Air-dried thalli were stored in polythene bags, at 7°C in the dark until required, no longer than two weeks. Samples of 10 g of air-dried thalli were floated on 25 ml 40 μM cycloheximide in 0.1 M Tris-HCl buffer, pH 9.15, for 16 hr at 26°C in the dark (Martin-Falquina and Legaz, 1984).

Preparation of cell-free extracts and purification procedure

After 16 hr incubation, thallus samples were washed with distilled water and then macerated with 12 ml acetone at room temperature for 5 min to remove lichen phenols (Vicente et al., 1983). Acetone was discarded and dry residues were newly ground with 12 ml 0.1 M Tris-HCl buffer, pH 9.15, containing 0.5 mM manganese sulfate and 0.75 mM maleic acid (Greenberg, 1955). The fresh homogenate was centrifuged at 24,000×g for 20 min at 4°C. Supernatant

was filtered through Millipore GS filters (0.22 μm pore diameter) and immediately afterwards ammonium sulfate was added to reach a final concentration of 70% (w/v). The mixture was stored for 2 hr at 4°C. The precipitate was discarded and the supernatant, containing the highest arginase activity, was dialyzed for 20 hr against 5.0 l of 10 mM Tris-HCl buffer, pH 6.5, at 4°C. Protein in the dialysate was then adsorbed on calcium phosphate gel, prepared according to Legget-Bailey (1967), 75 mg dry gel *per* mg protein. Arginase was eluted from the gel with increasing concentrations of Tris-HCl buffer, pH 6.5 (5 mM increments from 10 mM to 280 mM). The eluate from 160 mM contained the highest arginase activity.

Assay of enzyme activity

Arginase activity was assayed according to the Greenberg (1955) method as modified by Legaz and Vicente (1980) by including crystalline urease in the reaction mixtures. These contained, in a final volume of 3.0 ml, 10 μmol Tris-HCl buffer, pH 6.5, 0.4 μmol L-arginine, 7.5 μmol maleic acid, 5.0 μmol manganese sulfate, 8.1 mg crystalline urease and 5.0 μg lichen protein. Reaction was stopped by adding 0.5 ml of a saturated potassium carbonate solution. The amount of ammonia produced was measured by the Conway (1962) microdiffusion method. Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard. A unit of specific activity was defined as 1.0 μmol of ammonia produced *per* mg protein *per* min.

Isoelectrofocusing in liquid column

Isoelectric point (pI) of purified arginase was determined by a LKB 8100 column. The pH gradient was made with 1% (w/v) ampholines (Servalyt), pH 3.5–10, and the density gradient was made with sucrose. The sample was layered before pH gradient was formed. The cooling temperature was 4°C. Fractions of 2.0 ml were collected and assayed for arginase activity and pH value.

Estimation of molecular mass and kinetic constants

Molecular mass of purified arginase was estimated by SE-HPLC on a TSK-PW XL G5000 column (Legaz et al., 1990b), equilibrated with 75 mM Tris-HCl buffer, pH 6.5. The void volume (V_0) was estimated from the elution volume (V_e) of standard blue dextran 2000. Molecular standards were tyroglobulin (669 kDa), apoferritin (440 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa) and cytochrome c(12,3 kDa). Protein

in the eluate was monitored at 280 nm. The molecular mass of arginase was estimated by interpolating its elution parameter into a straight line made by plotting log molecular mass *versus* log V_e/V_0 of the standards (Fig. 1).
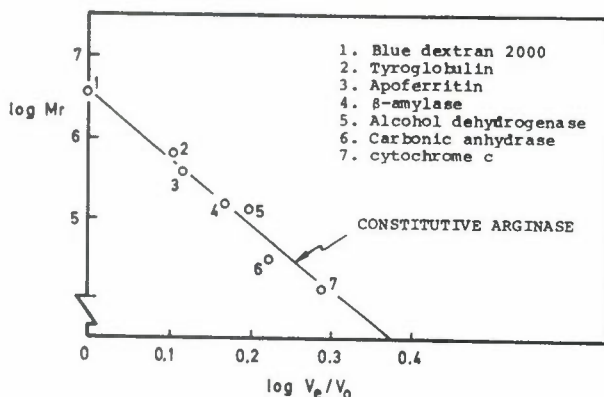

Figure 1. Determination of molecular mass of protein standards. Standards were: blue dextran 2000, tyroglobulin, apoferritin, β-amilase, alcohol dehydrogenase, carbonic anhydrase and cytochrome c.

Kinetic data were determined by varying both substrate and effector concentration in the reaction mixtures. Particular plots of these data were fitted by linear regression.

Binding analysis of evernic acid to arginase

Binding of evernic acid to purified constitutive arginase was achieved by equilibrium dialysis, at 26°C, for 30 min, in metacrylate half-cells separated by a semipermeable membrane. One of two compartments contained 1.0 ml of 2.68 μM arginase. The other contained 1.0 ml of evernic acid solution, the concentration of which varied from 0 to 50 μM (Table 1). Controls in which 1.0 ml Tris-HCl buffer substituted evernic acid solution were always performed.

After reaching equilibrium, evernic acid in both compartments was first extracted with diethylether:ethylacetate (65:35 v/v) and then analyzed by HPLC according to Legaz and Vicente (1983). Molecular mass of the protein after binding was estimated by SE-HPLC as above.

3. Results

Constitutive arginase was purified from *E. prunastri* thalli floated for 16 hr on 40 μM cycloheximide by conventional procedure including ammonium sulfate precipitation and adsorption chromatography with an overall yield of

Table 1. Estimation of the optimal times for equilibrium dialysis in the binding process of evernic acid to constitutive arginase

Time (min)	Evernic acid (mg ml^{-1})		
	Half-cell A_1	Half-cell A_2	Ratio A_1/A_2
2	0.048	0.113	0.43
45	0.072	0.102	0.70
90	0.078	0.095	0.82
150	0.079	0.092	0.85
180	0.085	0.084	1.01
225	0.083	0.084	0.98
285	0.087	0.089	0.97

The optimal time is that at which the $A_1/A_2 = 1$.

1.52% on the basis of total activity (Table 2). Fractions eluted with 160 mM Tris-HCl from calcium phosphate gel was electrofocused on a LKB column. Two main fractions display arginase activity, those with a pI value of 4.7 and 6.2 (Fig. 2). Fractions from sequential purification steps have also been analyzed by SE-HPLC. Figures 3a–c show the chromatograms obtained from cell-free extract, the supernatant from 70% ammonium sulfate precipitation and the eluate from calcium phosphate gel, respectively. The peak with a retention time of 30.3 min was identified as constitutive arginase. Purified arginase represents about a 94% of the total protein injected onto the column (Fig. 3c).

Substrate saturated kinetic showed a typical Michaelis-Menten relationship with a K_m value of 2.5 mM and a V_{max} equal to 48 nmol min^{-1} (Fig. 4) but the enzyme was inhibited by arginine concentrations higher than 6 mM. However, experimental plots of reaction rate *versus* substrate concentration when evernic acid was included in the reaction mixtures could be fitted either to sigmoid or exponential curves. The apparent K_m value in the presence of effector changed from 2.5 mM to 0.21 mM and V_{max} was equal to 1,800 nmol min^{-1}. The interaction coefficient, determined by Hill's plot, appeared to be $n_H = 1$ in the absence of the activator but it was somewhere between 3 and 4 in the presence of evernic acid (Fig. 5).

Since these kinetic data could be interpreted as a conformational change of the protein effected by evernic acid, studies on binding of this phenol to the protein were performed. Figure 6 shows the saturation kinetic of binding as a function of the concentration of total ligand. The curve obtained indicated

Table 2. Purification of the constitutive arginase of *Evernia prunastri*

| Step	Vol (ml)	Protein (mg)	Total protein (mg)	Specific activity (units)	Total activity (units)	Yield (%)	Purification (-fold)
Cell-free extract	150	8.55	1282.80	0.25	326.96	100	–
Supernatant from 70% ammonium sulfate	62	0.20	12.40	10.37	128.58	39.32	40.66
Fraction eluted with 0.16 M Tris-HCl buffer	20	0.12	2.40	30.50	73.34	22.43	119.84
Electro-focusing of pI = 6.18	3	0.025	0.07	66.06	4.95	1.52	259.05
Electro-focusing of pI = 4.68	3	0.060	0.18	11.25	2.03	0.62	44.12
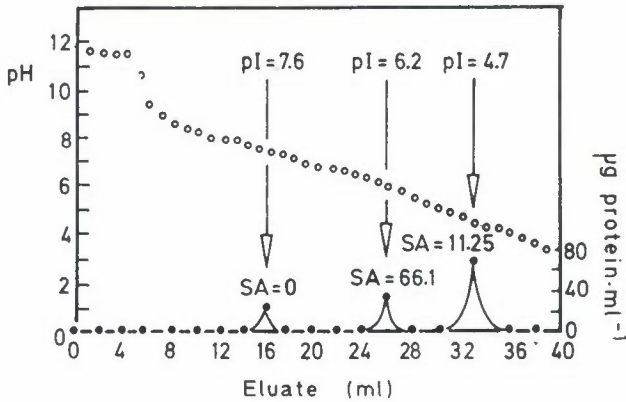
Figure 2. Electrofocusing of the purified constitutive arginase desorbed from calcium phosphate gel with 160 mM Tris-HCl buffer, (•) protein and (o) pH. SA is specific activity expressed as units and pI is isoelectric point of each fraction which contains protein.
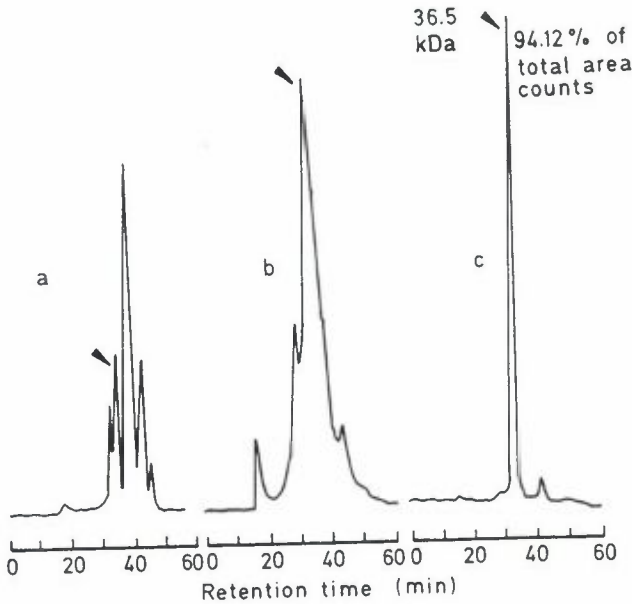

Figure 3. Chromatographic traces from SE-HPLC of (a) cell-free extract; (b) supernatant from ammonium sulfate precipitation and (c) fraction desorbed from calcium phosphate gel. Arrow shows the peak eluted at 30.3 min, identified as constitutive arginase.
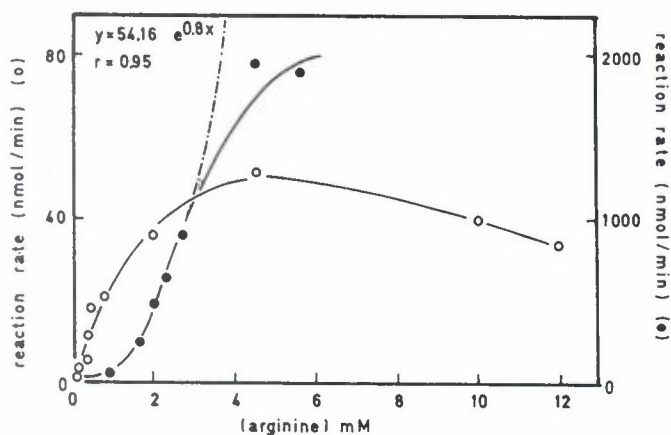
Figure 4. Substrate saturation kinetics of purified arginase without (o) and with (•) 2 nM evernic acid in the reaction mixtures.
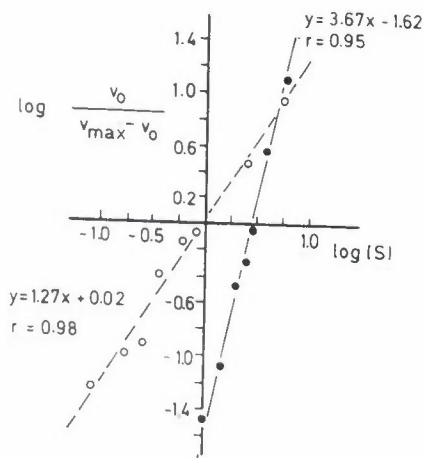

Figure 5. Hill plot to calculate interaction coefficient of constitutive arginase for its substrate. Symbols as in Fig. 4.

multiple sites for the ligand, this being in agreement with a possible aggregation of several enzyme subunits during the interaction process. This curve could be interpreted as sigmoidal because its double reciprocal plot shows an exponential function. Free ligand concentration *versus* fractional saturation of the binding sites (R^{-1}) showed clearly to be an exponential curve (Fig. 7). The intercept of this curve on Y axis, which represents R^{-1} (= [protein]/[bound evernic acid]) gave a n_H value of 3.26. Constitutive arginase appears to have 3 sites for evernic acid and shows a positive cooperativity to bind the ligand.

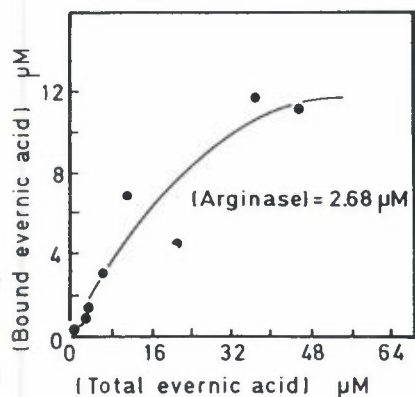To relate this binding behavior to the possible interconversions between

Figure 6. Kinetic of saturation of ever-
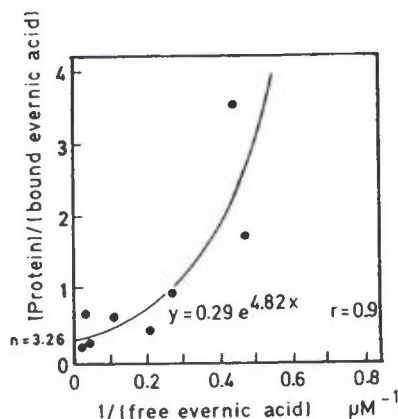nic acid binding to arginase
molecule.

Figure 7. Double-reciprocal plot of ev-
ernic acid binding to arginase
molecule to calculate the num-
ber of interaction sites for the
ligand.

different molecular mass arginases, both purified and effector-modified pro-
teins were analyzed by SE-HPLC. Purified protein contains about 62.4% of
a monomeric subunit with a molecular mass of 36.5 kDa (Fig. 8) whereas
arginase containing bound evernic acid decreases the amount of this monomer
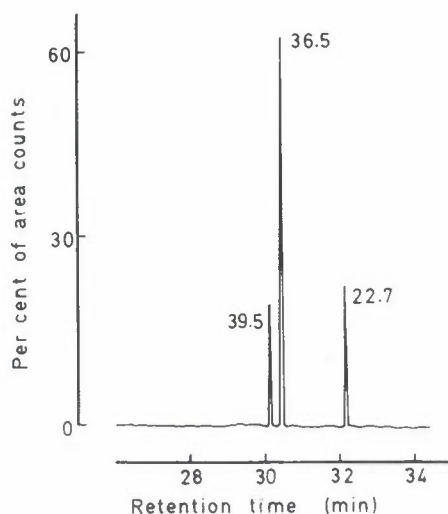to 5% by producing several polymers (Fig. 9).

Figure 8. Diagrammatic representation of the separation in SE-HPLC of different forms of
constitutive arginase which appears after equilibrium dialysis against Tris-HCl
buffer. The peak height symbolizes per cent of area counts and the numbers near
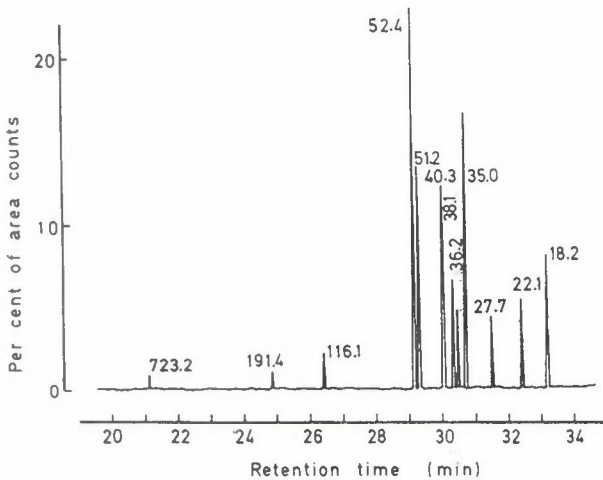the peaks are the molecular mass of each one, in kDa.

Figure 9. Diagrammatic representation of the separation in SE-HPLC of different forms
of constitutive arginase which appears after equilibrium dialysis against 40 μM
evernic acid. The peak height symbolizes per cent of area counts and the numbers
near the peaks are the molecular mass of each one, in kDa.

4. Discussion

Constitutive arginase is produced by *E. prunastri* thalli on 40 μM cyclo-
heximide. The fact that arginase activity develops in the presence of a trans-
lational inhibitor of protein synthesis is in agreement with that reported by
Weiss and Davis (1973, 1977) for a pre-existent arginase in *N. crassa*.

Purified constitutive arginase has at least two different subunits that can
be separated on the basis of their pI value, 4.7 and 6.2, respectively (Fig. 2).
SE-HPLC analysis also showed that the latter is resolved in two peaks. The
main one has a molecular mass of 36.5 kDa and is almost identical to that
reported for *N. crassa* (Borkovich and Weiss, 1987a) and mouse liver arginase
subunits (Spolarics and Bond, 1988).

Changes in the kinetic behaviour of constitutive arginase, produced by ev-
ernic acid (Fig. 4), are compatible with the role of evernic acid as an arginase
activator reported by Martin-Falquina and Legaz (1984). In addition, they
could imply a conformational change of the protein, effected by the ligand

(Price and Stevens, 1982). This possible effect is confirmed by the change in the number of interaction sites for L-arginine, from 1 to 3–4 (Fig. 5), produced after incubation of the enzyme with evernic acid. This last n_H value may indicate that constitutive arginase displays a positive cooperativity in the presence of the activator. That is, evernic acid produces a conformational change in the protein that increases the affinity of the enzyme for its substrate. This can be related to the work of Carvajal and Cederbaum (1986) concerning the manner in which regulatory sites on human liver arginase are related to molecular interconversions (Carvajal et al., 1982).

The ligand also binds to arginase following a clear pattern of positive cooperativity (Figs. 6 and 7) which is the consequence of a n_H value higher than 1 (Neet, 1980), and the evernic acid's three sites of interaction with the protein (Fig. 7). The saturation curve shown in Fig. 6 is sigmoidal, according to Price and Dwek (1983), since the double-reciprocal plot is an exponential curve. As a consequence of this positive cooperativity, the content of the native form of native purified arginase decreases from 62.4% to 5% (Figs. 8 and 9) whereas several polymeric and smaller forms appear (Fig. 9). It is interesting to note that the peak with a retention time value of 24.9 min corresponds to an oligomer of 36.5 kDa subunit, since its molecular mass has been determined as about 191 kDa. This oligomer represents about 1% of the total protein. The occurrence of trimers and hexamers also explains why the interaction coefficient of the protein for its substrate increases from 1 to 3 after incubation with evernic acid (Fig. 5). As shown in Fig. 9, not only polymers appear, but also molecular masses lower than 36.5 kD could be detected. About 8% of the total protein eluted from SE-HPLC column has a molecular mass of 18.2 kDa (retention time 33.2 min), similar to that postulated for the subunit of the induced enzyme (Legaz, this issue). This fact possibly implies that this last form was the true monomer of *Evernia* arginase. Then, the peak at 29.1 min, with a molecular mass of about 52.4 kDa, will be a trimer of this smallest subunit (Fig. 9). Polymerization-depolymerization of proteins by lichen phenolics has been reported by Vicente and Legaz (1988) for other lichen enzymes.

An open question would be then what is the quaternary structure of arginase under *in vivo* conditions, since equilibrium dialysis against Tris-HCl seems to dissociate a part of the possible dimer of 36.5 kDa to a smaller subunit of 22.7 kDa (Fig. 8). We may assume that other effectors, different from evernic acid, bind to the protein during purification and are partially removed during equilibrium dialysis. This effector is not evernic acid, since no phenol was removed from the control half-cell containing Tris-HCl buffer after equilibrium dialysis.

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