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OCCURRENCE AND SOME PROPERTIES OF CARBONIC ANHYDRASES FROM LEGUME ROOT NODULES

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Abstract—Carbonic anhydrase activity (hydration of CO_2) was found in homogenates of leaves (116–500 units mg^{-1} protein) and root nodules (27–255 units mg^{-1} protein) from 8 legume genera inoculated in each case with a host specific *Rhizobium* No enzyme, or only trace amounts (2–7 units mg^{-1} protein), were detected in root extracts The enzymatic activity was inhibited in all cases by azide and acetazolamide The sizes of nodule and leaf carbonic anhydrases, estimated by gel filtration of partially purified preparations from *Phaseolus vulgaris*, were around 45 000 and 205 000 respectively These enzymes also differed in sensitivity to inhibitors More than 99% of the activity present in *Vicia faba* nodules was recovered as a soluble enzyme and only a trace was located in the isolated bacteroids

INTRODUCTION

CARBONIC anhydrase (carbonate hydrolyase, E C 4 2 1 1) activity is readily detectable in leaf extracts from higher plants[1] and in algal homogenates [2] In C_3 plants more than two-thirds of the enzyme is associated with the chloroplast stroma[3,4] and this, together with a number of physiological observations,[5,6] has led to the implication that in plants, carbonic anhydrase functions in photosynthesis While the non-green areas from variegated leaves and albino leaves contain much less enzyme than normal green tissue,[7] no activity has been detected in root extracts [8,9] However, Bradfield[8] reported that "a small but distinct activity was found in extracts of nodules of French beans" The present study confirms Bradfield's observation and establishes the presence of significant carbonic anhydrase activity in the nodules but not in the roots of legumes The enzyme from bean nodules was partially purified and some of its properties compared with carbonic anhydrases from other sources

RESULTS

Substantial carbonic anhydrase activity was detected in the extracts of root nodules from all legume species examined (Table 1) Generally the specific activity was lower in

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TABLE 1 DISTRIBUTION OF CARBONIC ANHYDRASE ACTIVITY BETWEEN LEAF, ROOT, AND NODULE TISSUE FROM LEGUMES AND SENSITIVITY OF THE ENZYMES TO INHIBITORS

Source of enzyme	Carbonic anhydrase activity (units g^{-1} fr wt)	(units mg^{-1} protein)	% Inhibition by 2.5×10^{-5} M Azide	Acetazolamide
Vicia faba				
Leaf	5420	258	60	34
Root	Nil †			
Root tips*	Nil	—		
Primary nodules	1920	107	39	38
Lateral nodules	1740	77	42	35
V. sativa				
Leaf	16 150	397	68	34
Root	9	2		
Nodules	3060	96	48	42
Pisum sativum				
Leaf	7900	148	72	43
Root	Nil	–		
Nodules	3820	125	55	51
Glycine max				
Leaf	3250	116	71	34
Root	Nil			
Primary nodules	2490	55	35	73
Lateral nodules	1235	27	38	74
Trifolium repens				
Leaf	7670	365	62	47
Root	Nil			
Nodules	7690	190	30	45
Medicago sativus				
Leaf	20 200	500	72	49
Root	25	5		
Nodules	6600	255	50	47
Phaseolus vulgaris				
Leaf	14 000	471	78	49
Root	Nil	—		
Nodules	1525	49	56	90
Immature nodules	560			
Aged nodules	191			
Lupinus spp				
Leaf	10 200	430	78	47
Root	Nil	—		
Nodules	1730	77	50	71
Melilotus spp				
Leaf	2640	132	45	35
Root	57	7		
Nodules	3450	114	41	66

* The first 1–2 cm of root only

† Activity was not detectable in the assay

nodules than in leaves, although, on a fresh weight basis in *Trifolium* and *Melilotus*, and on a protein basis in *Pisum*, similar activity was extracted from both tissues

In the extracts of root tissue from which the nodules were removed little or no activity was found The traces from *Vi. sativa*, *Medicago* and *Melilotus* roots, could have been due to contamination by nodule tissue as, in these cases, the nodules were quite small and immature ones were difficult to excise completely The meristematic root tip tissue from *V faba* which was considerably enriched in protein (about 10 mg g^{-1} fr wt compared to 4 mg g^{-1} fr wt in whole roots) also failed to yield any detectable carbonic anhydrase The possibility that an inhibitor was present in the root extracts, so masking the enzyme, was tested by homogenizing equal weights of nodule and root tissue However, no effect of the added root was observed, suggesting that in the roots there was neither enzyme nor inhibitor

The enzyme was found in nodules formed on the primary root as well as on lateral roots (Table 1) and, even though immature nodules contained far less leghaemoglobin than the usual tissue extracted, they also exhibited a significant activity Aged nodule tissue, which was green in colour, had only 10% of the activity found in mature tissue (Table 1)

The enzymatic activity from both nodules and leaves was inhibited by acetazolamide and azide (Table 1) While in all cases the leaf activity was more sensitive to azide than to the sulfonamide, this was not so for the nodule extracts In fact the enzymes from *Glycine*, *Lupinus*, *Melilotus* and *Phaseolus* nodules were considerably more sensitive to acetazolamide Partially purified extracts were also inhibited by these compounds but all the plant enzymes were much less sensitive to the sulfonamide as compared with bovine erythrocyte carbonic anhydrase (Table 2)

TABLE 2 COMPARISON OF SIZE AND INHIBITION BY SODIUM ACETAZOLAMIDE AND SODIUM AZIDE OF DIFFERENT CARBONIC ANHYDRASES

Source of carbonic anhydrase	MW† ($\times 10^{-3}$)	I_{50} (M)‡ Acetazolamide	Azide
Bovine erythrocyte*	30	2.9×10^{-8}	2.3×10^{-4}
Hordeum leaf§	45	2.0×10^{-6}	2.2×10^{-4}
Phaseolus nodule	45	3.0×10^{-6}	1.6×10^{-5}
Phaseolus leaf	205	2.4×10^{-5}	4.6×10^{-6}

* A purified sample from Calbiochem
§ The plant enzymes were partially purified
† MW determined by gel filtration
‡ The same activity of enzyme from all sources was used and I_{50} is the concentration (M) causing 50% inhibition of activity

To compare the size of the nodule enzyme with other carbonic anhydrases, estimates of molecular weight were derived from the elution volume of each from a Sephadex G200 column *Phaseolus* nodule carbonic anhydrase was eluted with the same volume as the enzyme from *Hordeum* leaves This was between bovine serum albumin (MW 67 000) and α-chymotrypsinogen (MW 25 000), giving an estimate of size for both enzymes of around 45 000 daltons The *Phaseolus* leaf enzyme was considerably larger and eluted between catalase (MW 232 000) and alcohol dehydrogenase (MW 150 000), giving an estimate of around 205 000 daltons

Removing the bacteroids from a crude homogenate of *V faba* nodules did not significantly reduce the total activity of carbonic anhydrase in the extract (Table 3) In addition,

the isolated bacteroid fraction exhibited no activity, and though it contained about one-third of the soluble protein from the nodule an insignificant amount of carbonic anhydrase was liberated following disruption Passage of the soluble enzyme through the French pressure cell had no effect on the activity The data in Table 3 suggests that over 99% of nodule carbonic anhydrase was located outside the bacteroids

TABLE 3 LOCATION OF CARBONIC ANHYDRASE ACTIVITY IN NODULES FROM *Vicia faba*

Fraction*	Soluble protein (mg)	Total enzyme activity (units)
Nodule homogenate	75 0	10 240
Soluble extract	53 8	10 180
Bacteroids		Nil
Disrupted bacteroids	22 0	33

* The nodule homogenate was separated into bacteroids and a soluble extract by centrifugation and the bacteroids disrupted by passage through a chilled French pressure cell at $1\cdot04 \times 10^3$ kg cm^{-2}

DISCUSSION

The detection of significant carbonic anhydrase activity in root nodules from a wide range of legume-rhizobium symbioses confirms the initial observation of Bradfield[8] and establishes this enzyme as a normal constituent of active nodules Sensitivity to the sulfonamide acetazolamide, while lower than that of the animal enzymes suggests that like other plant carbonic anhydrases[9 11] the nodule enzyme is a Zn-protein

Origin of nodule carbonic anhydrase

The only well-characterized bacterial enzyme[12] (from *Neisseria sicca*) closely resembles the animal types in size and affinity for sulfonamides The comparison of these properties made in Table 2 shows that while the nodule enzyme was similar to the leaf types in sensitivity to inhibitors, the size was that of the monocotyledon rather than the typical dicotyledon type[10] found in bean leaves The apparent absence of activity in extracts of isolated bacteroids (Table 3) lends further support to the idea that the enzyme is of plant rather than bacterial origin

While in root tissue carbonic anhydrase activity appears normally to be repressed completely (or else the enzyme is inactive), in the process of nodule formation derepression (or activation) occurs In contrast, another nodule enzyme, nitrogenase is repressed in free living *Rhizobium*[13] but is synthesized by the bacteroids in the symbiosis That the enzyme formed in the nodule differs from the one in the leaves of the same plant can be explained if there is genetic information for both types, formation of the smaller predominating in the nodule and the larger in the leaf Some support for this explanation comes from the observation that trace amounts of a much smaller carbonic anhydrase have been found in dicotyledon leaf extracts following electrophoresis[1] Another possibility is that there is information for only one carbonic anhydrase protein and that this corresponds in size to the subunit of the dicotyledon enzyme[9 11] (*ca* 30 000 daltons and containing one atom

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Zn) Thus, while in the leaf a hexameric quaternary structure predominates, in the nodule there is little association of sub-units A further exploration of these possibilities awaits the purification and characterization of both enzymes

Physiological function of nodule carbonic anhydrase

Poincelot[4] has estimated that the level of carbonic anhydrase found in spinach leaf chloroplasts could theoretically support a rate of CO_2 hydration around 20 mmol mg chl^{-1} hr^{-1} Nodules contained on average one-third as much activity as legume leaves (Table 1) and so possess a lower but nevertheless considerable potential for this reaction

If, *in vivo* leghaemoglobin acts to facilitate the diffusion of O_2 to the respiratory sites in the bacteroid while maintaining the low internal free O_2 concentration necessary to prevent inactivation of nitrogenase (see Bergersen[13]), a function for carbonic anhydrase in nodule respiration may be envisaged Firstly, a high internal CO_2 concentration could cause formation of carbaminohaemoglobin which, in blood at least,[14] has a much reduced affinity for O_2 and could therefore impair O_2 transport and limit respiration As carbamino groups are not formed from HCO_3^- accelerated hydration by carbonic anhydrase would maintain a low free CO_2 level in the tissue The second possibility is that leghaemoglobin could behave like the blood protein, which, as an oxygenated anion, causes isohydric transport by accepting the proton formed in the hydration of CO_2 and unloading O_2 towards the respiratory sites At the surface closest to the atmosphere, O_2 loading would tend to deprotonate the pigment, the proton being used to dehydrate HCO_3^- so flushing CO_2 out of the tissue Carbonic anhydrase would serve to catalyse the hydration–dehydration at both ends of the O_2 gradient and in this indirect way function in the nitrogen fixing metabolism of the legume nodule

EXPERIMENTAL

Plant material Nodulated legumes were grown in vermiculite with a N-free Long Ashton culture soln[15] containing 5 6 ppm Fe as iron–EDTA and micronutrients including Co The seed was inoculated in each case with a specific *Rhizobium* culture before sowing *Trifolium repens* L was inoculated with *R trifolii* (1) *Vicia faba* L and *V sativa* L with *R leguminosarum* (1001), *Pisum sativum* L with *R leguminosarum* (1045), *Medicago sativa* L and *Melilotus* spp with *R meliloti* (2001), *Lupinus* spp (blue lupin) with *R lupini* (3211), *Glycine max* L Merr with *R japonicum* (3407) and *Phaseolus vulgaris* L with *R phaseoli* (3605) The numbers in parentheses refer to the strain catalogue number of the Rothamsted Rhizobium Collection The plants were grown in a green house with supplemental fluorescent lighting (20 000 lx) to provide a 16 hr day Barley (*Hordeum vulgare* L cv Union) was cultured in the same way except that 16 mM NO_3^- was included in the nutrient solution Plants were harvested after 4–5 wks and the roots washed free from vermiculite with cold tap H_2O

Extraction and assay of enzyme activity Samples (1–5 g fr wt) of detached nodules root tissue free from nodules or leaf, were taken and well washed with deionized H_2O at room temp All subsequent operations were done at 4–5° The tissue was ground for 2 min in a mortar and pestle with sand (1 1, w/w) and 2 vol of breaking medium (0 1 M Tris-H_2SO_4, pH 8 3 at 20° and containing 2 mM tetra Na-EDTA and 0 1 M 2-mercaptoethanol) and filtered through 2 layers of Miracloth The residue was re-extracted with one volume of breaking medium and the combined filtrates centrifuged at 1300 *g* for 10 min Samples of the supernatant (usually 10 μl leaf extract or 50 μl root and nodule extracts) were taken for determination of carbonic anhydrase activity by the colorimetric assay of Wilbur and Anderson[16] as used by Rickli *et al*[17] Five assays of active and boiled enzyme were done

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in each case and the mean value expressed in units[1] on a fr wt or soluble protein basis Following precipitation by TCA[18] to remove the mercaptoethanol soluble protein was assayed in homogenates by the method of Lowry *et al*[19]

Partial purification of enzymes Active carbonic anhydrase preparations were made from *Phaseolus vulgaris* and *Hordeum vulgare* leaves and partially purified by precipitation with $(NH_4)_2SO_4$ as described previously[10] for dicotyledon and monocotyledon leaf enzyme types The bean leaf preparation was purified 3 5-fold, with 90% yield of initial activity and contained 1721 units mg^{-1} protein Nodules (268 g fr wt) were detached from 4-week-old *P vulgaris* plants and homogenized with 2 vol of breaking medium in a Waring blendor Large material was removed by filtration through Miracloth and the clear red supernatant collected after removal of bacteroids and cortical cell fragments from the homogenate by centrifugation (13 700 *g* for 20 min) By adding 0 3 g $(NH_4)_2SO_4$ to each ml of this supernatant more than 95% of the initial carbonic anhydrase activity was precipitated Following centrifugation (13 700 *g* for 30 min) the supernatant which contained the leghaemoglobin was discarded and the pellet resuspended in 100 ml of breaking medium diluted 10-fold Insoluble material was removed by centrifugation This procedure gave a 4-fold purification with about 40% yield and a preparation which contained 182 units of enzyme mg^{-1} protein The nodule enzyme was stable to dialysis and when stored at 4 in a closed vessel retained full activity for 10 days

Molecular size estimation by gel filtration The partially purified plant extracts were dialysed for 12 hr against 2 changes of the column eluting buffer (a 10-fold dilution of the breaking medium with 10 mM Na_2SO_4 added) and applied to a Sephadex G200 column (2 6 × 28 cm) equilibrated with the same buffer The elution volume of activity was compared to those found with purified proteins of known MW (myoglobin α-chymotrypsinogen bovine serum albumin alcohol dehydrogenase catalase and apoferritin) Dextran 2000 was used to determine the void vol of the column Carbonic anhydrase activity in the eluate was assayed as above Myoglobin was estimated at 410 nm and all other proteins by absorption at 280 nm

Preparation of bacteroids Nodules from *V faba* (7 g fr wt) were homogenized in a Waring blendor with breaking medium and large cell fragments removed by centrifugation (300 *g* for 10 min) From this supernatant the bacteroids were collected by centrifugation (7000 *g* for 30 min) and the pellet washed free from carbonic anhydrase activity with fresh breaking medium The bacteroids were resuspended in 5 ml breaking medium and disrupted by passage through a chilled French pressure cell at $1 04 \times 10^3$ kg cm $^{-2}$

Inhibitors Solutions of NaN_3 and Na acetazolamide were prepared and tested in the standard carbonic anhydrase assay as described previously[10]

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