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PURIFICATION AND PROPERTIES OF CARBONIC ANHYDRASE FROM *CHLAMYDOMONAS REINHARDII*

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Abstract—Carbonic anhydrase (CA) was purified from the unicellular green alga *Chlamydomonas reinhardii*, and the purity of the preparation was established by gradient gel electrophoresis. The purified enzyme exhibited a MW of 165 000 and contained 6 atoms of Zn. The subunit MW, as determined by dodecyl sulfate electrophoresis, was 27 000. These results are consistent with a quarternary structure which is hexameric, each monomer containing 1 g atom of Zn. Like spinach CA, and in contrast to other oligomeric plant CAs, a sulfhydryl reducing agent is not needed to stabilize the enzyme. CO_2-hydrase activity was inhibited by both acetazolamide ($I_{50} = 7.8 \times 10^{-9}$ M) and sulfanilamide ($I_{50} = 1.3 \times 10^{-5}$ M), as well as by certain inorganic anions. The purified enzyme showed relatively weak esterase activity with *p*-nitrophenyl acetate but was an extremely effective esterase with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone as the substrate. Both esterase activites could be completely inhibited by adding acetazolamide. In its gross structural characteristics, the *C. reinhardii* enzyme resembles the CAs from higher plants. However, in its esterase activity and the inhibition by sulfonamides it is markedly different from plant CAs and bears more resemblance to erythrocyte CAs.

INTRODUCTION

Carbonic anhydrase, CA (EC 4.2.1.1), has been detected in several species of unicellular green algae and blue-green algae [1–5]. While no specific function is known for the enzyme in these organisms, a regulatory role in photosynthesis has been implicated. For example, in both green algae [1–4] and blue-green algae [5], CA levels are dependent on ambient CO_2 concentration. CA activity essentially disappears when cells are grown under high CO_2 concentrations. In studies with *Chlorella pyenoidosa* [6], a correlation has been shown between the rate of photosynthesis and the development of CA activity as cells adapt to low levels of CO_2. Activity of ribulose-1,5-diphosphate carboxylase and other enzymes associated with photosynthesis remained unchanged. Similar findings have been reported for *Chlorella vulgaris* [4].

In view of these indications that algal CA is adaptive with CO_2 and may, under certain conditions, regulate photosynthetic CO_2 fixation, it seemed important to us to investigate the molecular and catalytic properties of the enzyme. In addition, comparison of molecular data for the algal enzyme with data presently available for CAs from plants and animal sources may provide some insight into the phylogenetic history of this ubiquitous enzyme.

In this paper we report on the purification and some molecular and catalytic properties of CA from the unicellular green alga *Chlamydomonas reinhardii*.

RESULTS AND DISCUSSION

Purification

Prior to developing the purification procedure described (see Experimental), we investigated the effect of sulfhydryl reducing agents on CA activity in crude extracts of *C. reinhardii*. With the exception of the enzyme from spinach, all plant CAs that have been studied require a sulfhydryl reducing agent for stabilization, and such agents have been routinely added to protect the enzyme. We found that the *C. reinhardii* enzyme is stable in the absence of such agents and in fact, like spinach CA [7], loses activity in the presence of 2-mercaptoethanol. We also examined *C. reinhardii* homogenates for the presence of multiple forms of CA. Centrifugation of the homogenate at 20 000 *g* (40 min) yielded a supernatant which contained *ca* 90% of the total enzyme activity, and on polyacrylamide gradient electrophoresis showed a single band of enzyme activity which migrated *ca* 4 cm into the gel. When homogenate was not centrifuged, an additional diffuse, essentially non-mobile, band of activity was observed at the origin. We have not examined the pellet from centrifugation which may contain CA associated with particulate matter. Crude homogenate was routinely clarified by centrifugation as described.

The purification of the enzyme is summarized in Table 1. Typically, for each 10 l. of culture processed (*ca* 3×10^{10} cells) the procedure resulted in an 88-fold purification of enzyme from the homogenate supernatant and 16% of the activity in this supernatant was recovered. Recovery from the preparative electrophoresis step was low whether the enzyme was allowed to diffuse from macerated gel or was electrophoretically eluted from the gel. We continued to include this step in the procedure because of the favorable purification it affords and because it is the only means we have found to rid the preparation of a green contaminant. The material from preparative electrophoresis showed a single protein component when subjected to analytical polyacrylamide gradient electrophoresis.

Table 1. Purification of carbonic anhydrase from *Chlamydomonas reinhardii*

Fraction	Total protein* (mg)	Total activity (units)†	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
I Homogenate supernatant	991	109000	110	100	1.0
II $(NH_4)_2SO_4$	250	65000	260	60	2.3
III DEAE Bio-Gel A	78	43000	551	39	5.0
IV Sephacryl S-200	31	37500	1210	34	11.0
V Preparative electrophoresis	1.8	17500	9722	16	88.4

* Starting material, 10 l. of cell culture.

† Carbonic anhydrase activity was measured as the hydration of CO_2. The unit of enzyme activity is defined in the Experimental.

Molecular properties

Electrophoresis in a polyacrylamide gel gradient (4–30%) placed the molecular weight of the purified enzyme at an estimated value of 165000 ± 8000. The CAs from higher plants appear to consist of two main types: a high molecular weight type (MW 140000–190000) found in dicotyledons; and a low molecular weight type (MW *ca* 42000) found principally in monocotyledons [8]. Thus, in its molecular size the enzyme from *C. reinhardii* resembles the CA characteristic of dicotyledons.

Like all CAs isolated from higher plants, the enzyme from *C. reinhardii* is oligomeric. After dissociation and PAGE in the presence of SDS, the major component (*ca* 90%) in the preparation was a protein with a MW of 27000. As observed with pea leaf CA [8, 9], there was also a minor component exhibiting a MW of *ca* 50000. The minor component is probably the result of aggregation of the 27000 MW monomer as suggested for the enzyme from pea leaves. Consistent with findings for other CAs from both plant and animal sources, the enzyme from *C. reinhardii* contains zinc. For five samples of purified *C. reinhardii* CA the Zn content averaged 0.25%, corresponding to 1 g atom of Zn per 26000 of protein. This result coupled with the MW determinations (165000 before, and 27000 after dissociation) suggests a quarternary structure which is predominantly hexameric with each monomer containing 1 g atom of Zn, a structure also proposed for pea [9], parsley [10], spinach [11] and lettuce [12] CAs.

Inhibition of CO_2 hydrase activity

The results of inhibition studies are summarized in Table 2. The apparent similarity between *C. reinhardii* CA and the enzyme from higher plants does not extend to the inhibition by sulfonamides. The *C. reinhardii* enzyme behaves more like the erythrocyte CAs in this respect. For example, acetazolamide, a potent inhibitor of erythrocyte CAs ($I_{50} \cong 10^{-8}$) [13] is a much weaker inhibitor of the plant enzymes ($I_{50} \cong 10^{-5}$) [8–11]. A more striking example is that of sulfanilamide which is a moderate inhibitor of the erythrocyte enzymes ($I_{50} \cong 10^{-6}$) [13] but exhibits no detectable inhibition of spinach CA [14].

Table 2. Inhibition of *C. reinhardii* carbonic anhydrase

Inhibitors	I_{50} (M)
Acetazolamide	7.8×10^{-9}
Sulfanilamide	1.3×10^{-5}
NaN_3	3.9×10^{-5}
NaCl	5.6×10^{-2}

Data refer to the CO_2 hydration reaction. The concentration of inhibitor causing 50% inhibition (I_{50}) was obtained from plots of log inhibitor concentration vs observed per cent inhibition.

Esterase activity

With *p*-nitrophenyl acetate as the substrate, the purified enzyme exhibited a relatively weak esterase activity ($k_{enz} = 2.3\,min^{-1}$) compared to the activity of bovine CA (BCA) ($k_{enz} = 67.0\ min^{-1}$) and human CA-B (HCAB) ($k_{enz} = 29.4\,min^{-1}$). However, *C. reinhardii* CA was found to be a very effective catalyst in the hydrolysis of the cyclic sulfonate ester, 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone ($k_{enz} = 24.4 \times 10^4\ M^{-1}$/sec). For comparison, the values of k_{enz} obtained for BCA and HCAB with this substrate were 26.2×10^4 and $18.8 \times 10^4\ M^{-1}$/sec, respectively. With respect to these hydrolytic reactions, the *C. reinhardii* enzyme again behaves more like erythrocyte than plant CAs. The CAs from higher plants appear to lack the catalytic versatility which characterizes erythrocyte CAs. For example, none of the plant CAs examined has been found to catalyse the hydrolysis of *p*-nitrophenyl acetate. There has been no report regarding the activity of plant CA toward the sultone. However, we found that a partially purified spinach CA had only 1–2% of the sultone-esterase activity of the *C. reinhardii* enzyme when the two were compared on the basis of velocity in the hydrolysis of the sultone per CO_2 hydrase unit (v_{enz}/CO_2 unit).

Both of the observed esterase activities of *C. reinhardii* CA could be completely inhibited by adding acetazolamide. In the case of acetazolamide inhibition of the sultone hydrolysis, a Dixon [15] plot of the data (Fig. 1) characterizes the inhibition as non-competitive with an apparent dissociation constant, K_i, of 1.3×10^{-8} M (pH

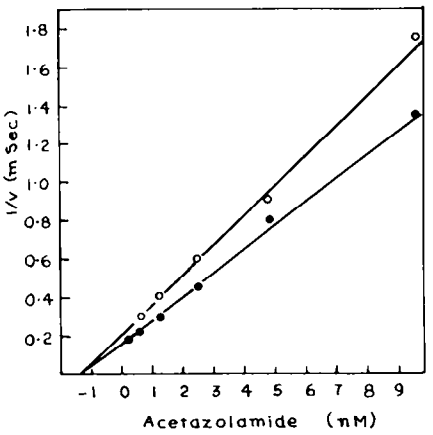

Fig. 1. Dixon plot for the inhibition by acetazolamide of the *C. reinhardii* CA-catalysed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone at $[S_o] = 84\,\mu M$, (○) and $[S_o] = 50\,\mu M$ (●). Velocities, obtained from first-order plots, were measured at 25° in 0.5 M Tris–SO_4, pH 7.7, 3% (v/v) acetone. $[E_o] = 2.6 \times 10^{-8}$ M.

7.4, 25°). In order to probe the stoichiometry of the reaction between enzyme and inhibitor, the data were treated by the method of Johnson *et al.* [16]. The slope of the straight line obtained was 1.06, indicating that acetazolamide forms a 1:1 complex with each active site on the enzyme. Similar stoichiometry has been observed for the reaction between acetazolamide and spinach CA, although the binding appears to be much less affined in the case of the spinach enzyme ($K_i = 2 \times 10^{-4}$ M) [17].

More detailed characterization of the weaker *p*-nitrophenylesterase activity, which could prove quite informative because K_m and k_{cat} are experimentally accessible with this substrate, will require the availability of a rather large amount of purified enzyme.

EXPERIMENTAL

Algal culture and harvest. Chlamydomonas reinhardii, wild type, mating type (+) cells were grown in sterile Tris–minimal phosphate [18] with the addition of penicillin (50 μg per ml). Filter-sterilized air was bubbled through the cultures which were maintained at 25 ± 2°, and routinely examined for bacterial contamination. Cool-White Sylvania fluorescent tubes (F 400 W) were used to provide continuous light with an intensity of 4000 lx at the level of the culture. When a culture reached a cell density of *ca* 3×10^6 cells/ml (mid log phase), the cells were harvested by centrifugation, washed once with 0.02 M Tris–SO_4, pH 8.0, and stored at −20°.

Enzyme purification. All steps were carried out at 5° unless indicated otherwise. Frozen cells from 10 l. of culture were thawed, suspended to 15% (v/v) in 0.02 M Tris–SO_4, 1 mM EDTA, pH 8, and broken in a pre-cooled French pressure cell at 1000 kg/cm². The resulting homogenate was centrifuged at 20 000 g for 40 min and the supernatant (fraction I) was subjected to $(NH_4)_2SO_4$ fractionation. The ppt. from the 30–65% satd fraction was suspended in *ca* 15 ml 0.02 M Tris–SO_4, 1 mM EDTA, pH 7.0 (buffer I), and dialysed 18 hr against 4 l. of the same buffer. The clear supernatant after centrifugation of the dialysate was termed fraction II.

Fraction II was chromatographed on a DEAE Bio-Gel A column (2.6 × 40 cm) which had been equilibrated with buffer I. CA was eluted with the equilibrating buffer and active fractions were pooled (fraction III) and concd by precipitation with $(NH_4)_2SO_4$ (0.5 g added to each ml). The ppt. was recovered by centrifugation, dissolved in and dialysed against 25 mM Tris–SO_4, 50 mM Na_2SO_4, 1 mM EDTA, pH 8.1 (buffer II). The dialysate was subjected to gel filtration on a column of Sephacryl S-200 (1.5 × 55 cm) equilibrated with buffer II and enzyme was eluted with the same buffer. Active fractions were pooled (fraction IV) and protein was salted-out and stored as a suspension in satd $(NH_4)_2SO_4$ adjusted to pH 8 by adding solid Tris.

For further purification, the ppt. from fraction IV was recovered by centrifugation at 20 000 g for 30 min, dissolved in 4 ml 94 mM glycine, 6 mM Tris, pH 8.3, and dialysed against the same buffer. The dialysate was subjected to prep. PAGE as previously described [19] for 32 hr at 20 mA. Following electrophoresis, a thin longitudinal slice of the gel was used to locate CA, which was detected as described in ref. [20]. Using this slice as a guide, the enzyme-containing zone was cut from the gel column and macerated in 10 ml 8% (w/v) sucrose in buffer I, for the elution of CA. The eluate was dialysed against buffer I, and concd to 1 2 ml using a Millipore Immersible Molecular Separator. The concentrate (fraction V) was stored at −20°.

Measurement of enzyme activity. CO_2-hydrase activity was assayed using an electrometric method based on that described in ref. [21]. The reaction mixture contained 2 ml 25 mM Veronal buffer, pH 8.25, *x* ml enzyme soln and 1 − *x* ml H_2O at 3°. Two ml of satd CO_2 soln at 3° was injected into the mixture by syringe and the decrease in pH was followed using a recorder attached to a pH meter and combination electrode. For the uncatalysed reaction, *x* ml of appropriate buffer was substituted for the enzyme soln. Units of enzyme activity were calculated from the time required to lower the pH from 8.0 to 7.5 using the usual formula [22]: $U = 10(t_b/t_e - 1$, where t_b and t_e are the times of the uncatalysed and enzyme-catalysed reactions, respectively. Using a sample of purified BCA, the units calculated as described were directly proportional to the amount of enzyme added.

Esterase activity was assayed with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone as the substrate as described [23]. The assay mixture contained 100 μl of the sultone in Me_2CO, a 10–25 μl aliquot of the CA soln to be measured and 0.05 M Tris–SO_4, pH 7.40, to a total vol. of 3.1 ml. The change in A_{410} was followed with a recording spectrophotometer at 25°. For the reasons given [23], k_{enz}, the apparent catalytic second-order rate constant, is used to indicate catalytic efficiency. Consistent with presentations for other oligomeric CAs [11, 24] and for more direct comparison with data for the monomeric erythrocyte enzymes, k_{enz} values for *C. reinhardii* CA are expressed as per mol of monomer (MW 27 000).

Esterase activity was also investigated using *p*-nitrophenyl acetate as the substrate as described in ref. [25]. Values of k_{enz} (mol substrate hydrolysed/min/mol enzyme monomer) were obtained from initial velocities at 25° in 0.05 M Tris–SO_4, pH 7.7, 3.3% Me_2CO, $S_o = 1.36$ mM.

When studying the inhibition of CO_2 hydrase and esterase activities, appropriate amounts of inhibitor were mixed with enzyme and allowed 15 min to reach equilibrium prior to initiation of the reaction.

Protein was determined by the Lowry method [26]. Crystalline BSA served as the reference protein.

Analytical gel electrophoresis. Analytical gradient PAGE and the location of CA activity after electrophoresis were performed as described [20] using Pharmacia PAA 4/30 gradient gel slabs (4–30% concave gradient of acrylamide). Gels were stained for protein with Coomassie Brilliant Blue R [27]. The MW of *C. reinhardii* CA was estimated as described [12] using as ref. proteins: apoferritin (MW 460 000), catalase (MW 240 000),

lactate dehydrogenase (MW 140000) and BSA (MW 67000). SDS-PAGE, used to estimate the number and size of subunits, was performed as described [27].

Measurement of Zn. The Zn content of the purified enzyme was determined spectrophotometrically [28]. Using this method, the Zn content of purified human erythrocyte CA-B was found to be 1 g atom Zn per 29188 g protein.

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