Isoenzymic forms of carbonic anhydrase in the red macroalga Porphyra leucosticta

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Different isoenzymes of carbonic anhydrase (CA; EC 4.2.1.1) have been separated using thalli of the red macroalga Porphyra leucosticta Thuret in Le Jolis. Homogenates of the thallus were centrifuged in order to separate soluble and membrane proteins. The fraction containing membrane proteins was subdivided by centrifuging into two fractions: green and nongreen membrane proteins. CA activity was detected in all the fractions. Because external CA (measured on intact thallus) represented 15% of total activity, it was concluded that most of the CA (ca. 80%) was soluble and internal. Direct evidence regarding the different function of external and internal CA was obtained by determining the effects on photosynthesis of two specific CA inhibitors with different capacity for entering cell. It was concluded that internal CA was necessary to ‘trap’ the CO₂ entering the cell and thus maintain a favorable CO₂ gradient that permits its diffusive entry. Changes in the O₂ evolution rate at inorganic carbon (Cᵢ) concentration saturating for photosynthesis and on membrane proteins. The fraction containing membrane proteins was subdivided by centrifuging into two fractions: the photosynthetic conductance for Cᵢ were found when external CA was inhibited. Based on these changes and the significant CA activity (ca. 9% of the total activity) found in nongreen membrane fraction, the presence of external CA associated with plasma membrane was postulated. The presence of CA associated with chloroplast membrane was also suggested.

Introduction

The carbonic anhydrase enzyme (CA; EC 4.2.1.1) is required for functioning of the carbon-concentrating mechanism found in almost all the marine macroalgae studied (Smith and Bidwell 1989, Björk et al. 1992, Haglund et al. 1992a, García-Sánchez et al. 1994). It is accepted that there are two fractions of CA, external and internal (Giordano and Maberly 1989, Surif and Raven 1989, Björk et al. 1992, Haglund et al. 1992b, Sültemeyer et al. 1993). External CA is necessary for transforming HCO₃⁻ into CO₂ to be used for photosynthesis. Different CA isoenzymes have been characterized in green microalgae: mitochondrial 22-kDa CA (Eriksson et al. 1996), periplasmic 38-kDa CA (Coleman 1991) and chloroplastic 36-kDa CA (Ramazanov et al. 1993) have been described in Chlamydomonas reinhardtii. Fisher et al. (1996) demonstrated a plasma membrane of 60-kDa CA in Dunaliella salina. Internal location of a 24–25-kDa CA in higher plants has been demonstrated in chloroplast of C₃ plants (Fawcett et al. 1990) or cytoplasm of C₄ plants (Burnell et al. 1990). Biochemical data for macroalgae are scarce. Evidence for the presence of internal CA in macroalgae comes from: (1) comparison of the CA activity on the thallus and in the crude extract and (2) use of the CA inhibitors with different capacity for penetrating inside the cells (Sültemeyer et al. 1993). This evidence is not sufficient to define the exact location of the enzyme in macroalgae. Therefore, the proposed functions for the CA are controversial, especially for the external CA. In fact, no correlation was found by Mercado et al. (1998), between external CA activity and capacity for HCO₃⁻ use among 16 macroalga species. However, external CA activity was necessary for photosynthesis in almost all these species. These authors proposed different functions for the external CA that could be related to its location on the cell surface. Two locations are possible: associated with plasma membrane and/or attached to the cell wall (Sültemeyer et al. 1989, Björk et al. 1992, Fisher et al. 1996).

Abbreviations – AZ: acetazolamide; CA: carbonic anhydrase; EZ: ethoxyzolamide; gₚ: photosynthetic conductance for Cᵢ; Kₛ: apparent half-saturation point for Cᵢ; V_max: maximal photosynthetic rate at Cᵢ saturation; PE: phycoerythrin.
The aim of this study was to separate different isoenzymes of CA in the red macroalga Porphyra leucosticta. For this proposal, soluble and membrane proteins were separated and their CA activities were measured. In addition, the effect of CA inhibitors on photosynthesis was determined. A tentative model of the CA system based on biochemical and kinetic data is discussed.

Materials and methods

Plant material and culture conditions

P. leucosticta Thuret in Le Jolis was collected in the supralittoral zone near Lagos (Málaga, southern Spain) during winter 1996. After collection, the alga was maintained in a controlled-temperature room (15°C) at 12 h light day−1 for not more than 10–12 days. The thalli were stored in plexiglas cylinders containing 3 l of natural seawater. The medium was vigorously aerated (about 3 l air min−1). A constant photon fluence rate of 60 μmol m−2 s−1 was provided with day light fluorescent lamps (F20W/DL Osram, Germany). The photon fluence rate was derived by means of a quantum spherical PAR sensor (193SB; Li-Cor Inc., Lincoln, USA) connected to a radiometer (Li-1000; Li-Cor Inc., Lincoln, USA).

Oxygen evolution and effect of inhibitors of CA

Oxygen evolution was measured in small (8–9 ml) temperature-controlled (15°C) chambers equipped with a Clark-type oxygen microelectrode (Yellow Springs Instrument Co., Inc., Yellow Springs, OH, USA) at a photon fluence rate of 350 μmol m−2 s−1. Oxygen electrode tracings were recorded on a strip chart recorder. From 6 to 8 algal disks (ca. 40 mg) were transferred to the electrode chambers containing natural seawater buffered to pH 8.7 with 50 mM (ca. 40 mg) were transferred to the electrode chambers containing natural seawater buffered to pH 8.7 with 50 mM Tris buffer (2 M in stock). The effects of acetazolamide (AZ) and ethoxyxolamide (EZ) (Sigma-Aldrich Quimica S.A., Madrid, Spain) on O2 evolution were determined. It is generally assumed that AZ cannot penetrate into the cell and only inhibits the extracellular CA (Sültémeyer et al. 1993, Mercado et al. 1997a). Ethoxyxolamide penetrates into the cell and inhibits both external and internal CA (Sültémeyer et al. 1993). CO2-free stock solutions of the inhibitors were prepared in 0.05 M NaOH. The inhibitors were added at a final concentration of 100 μM.

The effects of the CA inhibitors were also tested under disequilibrium conditions of the carbonic-carbonate chemical system. For this study, algal disks were incubated in the electrode chambers containing a simple synthetic seawater medium without inorganic carbon (450 mM NaCl, 30 mM MgSO4, 10 mM KCl and 10 mM CaCl2, pH 8.7; Beer et al. 1990). The disks of thalli were briefly washed in the synthetic seawater before being enclosed in the electrode chambers. After a close-to-zero net O2 exchange rate had been reached, ice-cold CO2-saturated distilled water (CO2 concentration of 40 mM) was added. The O2 evolution was recorded during the next 15–20 min, whereupon AZ was applied. When a constant O2 evolution was obtained, CO2-saturated distilled water was again added. Oxygen evolution was recorded until stabilization. Afterwards, EZ was tested following the same procedure. Different oxygen tracing curves were generated by adding different amounts of CO2. The final CO2 concentration reached in the medium after CO2 additions ranged from 0.04 to 2.6 mM.

Oxygen electrode tracings were analyzed as follows. Calculations of the spontaneous CO2 hydration rate (according to Johnson (1982)) revealed that 90% of the added CO2 would be converted into HCO3− within 3–4 min when external CA was inhibited. Therefore, the O2 evolution obtained immediately after the CO2 addition in the presence of AZ was considered to be primarily due to CO2 diffusive entry, whereas the constant O2 evolution, obtained after 8–10 min, was due to HCO3− use. The photosynthetic rate obtained immediately after adding CO2 (i.e. under disequilibrium conditions) was calculated from the initial slope of O2 electrode tracing just after CO2 addition. O2 evolution rates obtained in the absence and presence of AZ were compared.

Photosynthetic rates obtained were plotted against CO2 concentration reached in the medium after CO2 additions. Photosynthetic rate versus CO2 concentration curves (P-CO2 curves) obtained in the absence and presence of AZ were fitted to a Michaelis-Menten model. Maximal photosynthetic rate (Vmax) and half-saturation point (Ks) were obtained from the model. The goodness of fit was tested by using least-squares regression analysis. P-CO2 curves were carried out in triplicate. Conductance for CO2 was also calculated from the initial slope of the curve. A value of 5.91 for fresh weight (unit surface area)−1 was used in calculating the O2 evolution rate (surface unit)−1 (Figueroa et al. 1995).

Extraction and fractionation of algal material

Plant material (20 g) was ground in extraction buffer (50 mM Tris, 25 mM isoascorbic acid, 25 mM mercaptoethanol, 5 mM EDTA) to 50 ml final volume. The crude homogenate was centrifuged at 250 g for 5 min in order to eliminate the debris from broken cells that formed a pellet at the bottom of the tubes. The homogenate was centrifuged (Haglund et al. 1992b) at 150000 × g for 1 h in a XL-90 Beckman Ultracentrifuge. The supernatant from this centrifugation contained the soluble proteins (Fraction 1). The pellet containing membrane proteins was resuspended in 30 ml of buffer and spun at 10000 g for 10 min. According to Haglund et al. (1992b), the supernatant contained nongreen membranes (Fraction 2) and the new pellet contained green membranes (Fraction 3). The new pellet was resuspended in 30 ml of buffer. A total of 5 ml of each fraction and homogenate was saved for the determination of CA activity. An absorbance spectrum of the fractions in the band 400–750 nm was derived with a Beckman DU-7 spectrophotometer. Protein concentration of the samples was determined in triplicate by means of the Coomassie blue G-250 method (Bradford 1976). Concentration of phycoerythrin (PE) in the fractions was derived according to Beer and Eshel (1985).

The process of extraction and fractionation was twice repeated, starting with independent samples. The results obtained were not significantly different.
Assay of carbonic anhydrase

CA activity was measured potentiometrically at 0–2°C by determining the time taken for a linear drop of 1.0 U of pH ranging from 8.5 to 7.5 (Haglund et al. 1992b). A vessel containing 3 ml of extraction buffer was used. External CA activity was assayed by using 60–90 mg of alga transferred directly from the culture to the vessel. The reaction was started by introducing 1 ml of ice-cold CO₂-saturated distilled water. One unit of relative enzyme activity (REA) was defined as \( \frac{t_0}{t_c} - 1 \)

\( t_0 \) and \( t_c \) were the times for pH change of the nonenzymatic (buffer) and enzymatic reactions, respectively. The activities in homogenate and fractions were determined as the mean value of 4–6 samples. Activity was assessed by applying EZ (50 \( \mu \)M) as a control.

**Statistics**

The results were expressed as the means ± SD. Statistical significance of means was tested with a model 1 one-way ANOVA followed by a multirange test by Fisher’s protected least significant difference (Sokal and Rohlf 1981).

**Results**

The oxygen evolution rate in natural seawater buffered at pH 8.7 (\( C_i \) concentration of 2.1 mM) was reduced by 70% when AZ was added (Fig. 1). It must be noted that the inhibition by AZ was almost instantaneous after its addition and that the effect remained constant through time. EZ was added in the presence of AZ. The addition of EZ produced a more pronounced inhibition of the oxygen evolution (the photosynthetic rate was close to zero).

An example of the oxygen electrode tracing obtained in \( C_i \)-free synthetic seawater medium buffered at pH 8.7 is shown in Fig. 2. The addition of CO₂ caused a rapid increase in \( O_2 \) evolution that remained constant through time indicating that CO₂ and HCO\(_3\)⁻ were used with identical affinity. However, following addition of AZ, a rapid inhibition of the steady state rate of \( O_2 \) evolution was obtained. The addition of CO₂, after AZ was added, produced an increase in the rate of \( O_2 \) evolution, although no \( O_2 \) evolution was observed after 5–6 min. Following stabilization of the \( O_2 \) evolution (after ca. 15 min), addition of EZ produced an slightly negative oxygen evolution. When CO₂ was added again (in the presence of EZ), \( O_2 \) evolution increased rapidly and declined after 5–6 min. It must be noted that the total amount of \( O_2 \) involved after CO₂ addition was lower than it was in the presence of AZ.

Fig. 3 shows the photosynthetic rate obtained just after addition of CO₂ plotted against \( C_i \) concentration reached in the medium (\( P-C_i \) curves). Photosynthetic rates followed typical saturation kinetics both in the absence and in presence of AZ. However, when external CA was inhibited with AZ, maximal photosynthetic rate was two-fold higher than without AZ (Table 1). Photosynthetic conductance estimated from the initial slope of the \( P-C_i \) curves was also increased from \( 34 \times 10^{-6} \) to \( 62 \times 10^{-6} \) m s⁻¹ in the presence of AZ. It must be noted that both \( K_c \) and \( V_{max} \) were modified when external CA was inhibited.

The amount of protein and CA activity obtained after centrifugation steps is summarized in Table 2. Additional evidence concerning the quality of soluble and membrane protein fractions was obtained by examining the absorbance spectrum of each fraction and spectrophotometrically measuring PE concentration (Fig. 4). PE was used as a marker for soluble proteins because it represents around 5% of total soluble protein in \( P. \) leucosticta (Figueroa et al. 1995). PE concentration in homogenate and soluble fraction was 41.4 and 65.58 \( \mu \)g mg⁻¹ of protein, respectively (Table 2). PE was undetectable in the fractions containing proteins associ-
Fig. 3. Photosynthetic rate reached just after CO2 addition versus \( C_i \) concentration curves. The highest maximal CO2-dependent photosynthetic rate was obtained after AZ addition, i.e. when external CA was inhibited. The curves were carried out in triplicate and fitted the Michaelis-Menten model. Bars represent SD (n = 3).

CA activity was inhibited. The curves were carried out in triplicate and fitted the Michaelis-Menten model. Bars represent SD (n = 3).

with membranes. From these results, it can be concluded that Fraction 1 contained most of the soluble proteins and that the level of contamination with soluble proteins in the fractions containing proteins associated with membranes was not detectable. Fraction 3 had a very strong absorbance peak for Chl a (678 nm) and carotenoids (480 nm). An absorbance peak for Chl a was absent in Fraction 1 but it appeared in Fraction 2. However, absorbance for Chl a relative to protein concentration was ca. 50-fold higher in Fraction 3 than in Fraction 2. Because Chl a and carotenoids are markers for proteins associated with thylakoids and chloroplast envelope, respectively (Palmqvist et al. 1990b, Haglund et al. 1992b), it can be concluded that Fraction 3 was strongly enriched in proteins associated with chloroplast membranes. Fraction 2 had to be enriched in nongreen membrane proteins.

All fractions contained CA activity (Table 2) that could be inhibited with EZ. Most of the CA activity was obtained in the soluble protein fraction (80%), although some activity was found also in the fraction containing green membrane proteins (8%) and in the fraction containing nongreen membrane proteins (9%). No significant difference (P > 0.05) among specific activity measured for the different fractions was found. Because specific

Table 1. Maximal CO2-dependent photosynthetic rate (\( V_{\text{max}} \)), half-saturation point (\( K_C \)) and photosynthetic conductance for \( C_i \) (\( g_p \)) were obtained from CO2-dependent photosynthetic rate versus \( C_i \) concentration curves. The curves were performed before adding AZ (Without AZ) and after adding the inhibitor (With AZ). The parameters were derived from the Michaelis-Menten model. The goodness of fit was tested by using least-squares regression analysis (P < 0.001). Data are means ± sd from three curves.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Without AZ</th>
<th>With AZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{max}} ) (( \mu \text{mol O}_2 \text{ m}^{-2} \text{ min}^{-1} ))</td>
<td>119.5 ± 17.92</td>
<td>226.5 ± 19.6</td>
</tr>
<tr>
<td>( K_C ) (mM)</td>
<td>1.90 ± 0.49</td>
<td>0.80 ± 0.16</td>
</tr>
<tr>
<td>( g_p ) (m s(^{-1}) ( \times ) 10(^{-6} ))</td>
<td>0.34</td>
<td>8.62</td>
</tr>
</tbody>
</table>

CA activity in the fraction containing soluble proteins was similar, but the protein content was qualitatively different, it must be concluded that the activities detected in the different fractions were due to different CA isoenzymes.

Discussion

CA system in Porphyra leucosticta

The possible location of CA in different cell compartments suggests that P. leucosticta possesses a complex CA system similar to that described for other well-studied green microalgae (Ramazanov et al. 1993, Fisher et al. 1996). In P. leucosticta, most activity has been found in the soluble protein fraction, indicating that this alga contains soluble CA. It is not possible to specify the exact location of the soluble CA from these experiments. However, taking into account that the external CA activity represents ca. 15% of total activity (Mercado et al. 1997a), most of the soluble CA activity must be located inside the cell. At least three locations for soluble CA are possible: cytosol; stroma of chloroplast and cell wall. Soluble cytosol and stroma CA have been observed in C. reinhardtii and other green microalgae that have a carbon-concentrating mechanism (Spalding et al. 1983, Sültemeyer et al. 1989). Some other authors have demonstrated soluble CA activity attached to cell wall by ionic interactions in microalgae (Aizawa and Miyachi 1984, Sültemeyer et al. 1989). Palmqvist et al. (1990a) and Ramazanov et al. (1993) demonstrated the presence of CA activity associated with membrane systems for different green microalgae. Haglund et al. (1992b), following the same procedure described in this paper, demonstrated CA activity associated with membranes from a red macroalga, Gracilaria tenuistipitata. From the above results, the presence of CA activity tightly associated with membranes can be also postulated for the red macroalga P. leucosticta.

Evidence for the presence of external and internal CA isoenzymes from the use of the inhibitors is also in this paper. The presence of external CA was assessed by determining the effect of AZ (specific CA inhibitor unable to cross the plasma membrane). Some authors argue against the use of AZ as a proof of external CA presence because the inhibitor may enter the cell (Williams and Turpin 1987, Smith and Bidwell 1989) and thus inhibit internal CA. This possibility can be discounted based on the above data, because the effects of AZ and EZ on O2 evolution were distinguishable: firstly, an additional inhibition of photosynthesis rate by EZ (an inhibitor that can penetrate into the cell) was detected in natural seawater containing added AZ; secondly, photosynthetic rate, dependent on CO2 diffusive entry (obtained just after addition of CO2 under disequilibrium conditions) was lower when EZ was applied in the presence of AZ. Any AZ entering the cell would counteract these differences. In addition, there was no time dependence on the effect by AZ (if AZ penetrates slowly into cells then an increase on photosynthetic inhibition through time is expected).
Table 2. Specific (REA mg\(^{-1}\) protein) and total (REA) carbonic anhydrase activity and total protein (mg) and phycoerythrin content (\(\mu\)g mg\(^{-1}\) protein) in the homogenate and the three different fractions obtained after centrifugation steps. The numbers are the results of one experiment. The experiment was repeated twice with similar results. (Data are means ± SE; n = 4). ND, not detected.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Homogenate</th>
<th>Soluble</th>
<th>Membrane fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nongreen</td>
</tr>
<tr>
<td>Specific activity</td>
<td>0.25 ± 0.03</td>
<td>0.27 ± 0.10</td>
<td>0.27 ± 0.11</td>
</tr>
<tr>
<td>Total activity</td>
<td>34.6 ± 4.8</td>
<td>29.4 ± 11.0</td>
<td>3.12 ± 1.27</td>
</tr>
<tr>
<td>Total protein</td>
<td>138.4</td>
<td>109.6</td>
<td>11.54</td>
</tr>
<tr>
<td>PE</td>
<td>41.4</td>
<td>65.6</td>
<td>ND</td>
</tr>
</tbody>
</table>

It is not easy to obtain direct evidence of the role of the different CA isoenzymes on \(C_4\) acquisition in macroalgae. In this paper, the use of inhibitors under disequilibrium conditions evidenced the implications of external and internal CA on HCO\(_3^-\) use in \(P.\) leuconostica. Mercado et al. (1997a) demonstrated that \(P.\) leuconostica lacks a mechanism for direct HCO\(_3^-\) uptake. Therefore, the function of the external CA at alkaline pH must be to permit the use HCO\(_3^-\) by means of its transformation into CO\(_2\) (Mercado et al. 1997a,b). The amount of O\(_2\) involved when internal CA inhibited (with EZ) was lower by 40% than when only external CA was inhibited (with AZ). From this result, it can be postulated that the internal CA is necessary to ‘trap’ the CO\(_2\) entering the cell, probably by accelerating its transformation into HCO\(_3^-\). The high level of soluble CA activity (probably located in the cytoplasm and/or stroma) could be necessary to maintain a favorable CO\(_2\) concentration gradient that permits the CO\(_2\) diffusive entry.

**External CA as an active sieve for CO\(_2\) diffusive entry**

The proposed function for the external CA appears in contradiction with the results obtained from the experiments carried out under carbonic-carbonate system disequilibrium conditions because the CO\(_2\) diffusive entry increased when external CA was inhibited, i.e. external CA apparently acted as a sieve for the CO\(_2\) diffusive entry. Two hypothesis can explain this result: (1) According to the kinetics of the reaction performed by CA, the high levels of external activity in \(P.\) leuconostica cause all forms of inorganic carbon close to the plasma membrane to be immediately equilibrated with one another. Thus, the CO\(_2\) added will be transformed into HCO\(_3^-\) more rapidly when external CA is activated and then a lower CO\(_2\) gradient is reached (equivalent to a lower O\(_2\) evolution rate). However, this effect can not explain fully the above results because the kinetics for the CO\(_2\) diffusive entry (with AZ) and that mediated by external CA (without AZ) were different. It would be expected to obtain similar \(V_{\text{max}}\) and higher \(K_s\) when \(P-C_i\) curve is performed without AZ, but both \(V_{\text{max}}\) and \(K_s\) were modified when external CA was inactivated; (2) A complementary explanation is that external CA does not act as a mere passive sieve for CO\(_2\) but it could also modify the conductance for CO\(_2\) of the plasma membrane. In this way, the differences on \(V_{\text{max}}\) at \(C_i\) saturation and \(g_p\) can be explained. The presence of CA associated with the plasma membrane is necessary to support this hypothesis. A similar hypothesis has been proposed by different authors: Tsuzuki and Miyachi (1989) assumed the cooperation of CA and an active transport system for free CO\(_2\) in order to explain the accumulation of inorganic carbon within algal cells; Volokita et al. (1984) suggested that a membrane transport system of HCO\(_3^-\) involves a CA-like moiety in \(Anabaena\).

The exact location of the external CA in macroalgae is not clear. Beer and Björk (1994) found that protoplasts of \(U\) \(c\) \(h\) \(a\) \(c\) \(i\) \(t\) \(a\) did not display activity of extracellular/surface-bound CA. Regarding microalgae, some authors have demonstrated that external CA is attached to cell wall in \(C.\) reinhardtii (Sültemeyer et al. 1989, Coleman 1991). In the same sense, the mutant \(C W\) 92 of \(C.\) reinhardtii lost external CA at 90% of wild type indicating that it is associated with the cell wall but not with the external surface of plasma membrane (Palmqvist et al. 1990a). In contrast, plasma membrane CA isolated by Fisher et al. (1996) in Dunaliella salina is a surface enzyme. From the above results, plasma membrane CA in \(P.\) leuconostica can be postulated because CA activity was detected in the fraction containing non-green membrane proteins with a probable high content of plasma membrane (Palmqvist et al. 1990b, Haglund et al. 1992b).

![Fig. 4. Absorbance spectra of the algal homogenate and subfractions. Chl \(a\) absorption peak (678) was absent in Fraction 1 although this fraction was enriched in PE. Fraction 3 has a very strong absorbance peak for Chl \(a\) and carotenoids. From these results, it was concluded that Fraction 1 was enriched in soluble protein, Fraction 2 in non-green membrane protein and Fraction 3 in green membrane protein.](image-url)
In conclusion, the presence of different CA isoenzymes in *P. leucosticta* is demonstrated. Kinetic and biochemical data suggest the presence of external CA is associated with plasma membrane. It could be necessary to permit the use of HCO$_3^-$ by catalyzing its transformation into CO$_2$ and its entry into the cell. Soluble internal CA could be necessary to ‘trap’ CO$_2$ into the cell. In addition, the presence of CA activity associated with chloroplast membrane is suggested.

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**References**


Dunaliella salina, J Biol Chem 271: 17718–17723


Mercado JM, Niell FX, Figueroa FL (1997a) Regulation of the mechanism for HCO$_3^-$ use by the inorganic carbon level in *Porphyra leucosticta* Th. in Lell Jollii (Rhodophyta). Plan 201: 319–325


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