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Identification of Intracellular Carbonic Anhydrase in *Chlamydomonas reinhardtii* with a Carbonic Anhydrase-Directed Photoaffinity Label[1]

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A carbonic anhydrase (CA)-directed photoaffinity reagent, ^{125}I-labeled *p*-aminomethylbenzenesulfonamide-4-azidosalicylamide, was synthesized and shown to derivatize periplasmic CA in the unicellular green alga *Chlamydomonas reinhardtii*. The photoderivatization of purified *C. reinhardtii* periplasmic CA or intact *C. reinhardtii* cells with the reagent resulted in the modification of the large (37 kD) subunit of the enzyme. Photoderivatization of proteins in lysed *C. reinhardtii* cells also resulted in the specific labeling of a polypeptide of 30 kD. Centrifugation of the cell extract prior to photoaffinity labeling revealed that the labeled peptide was present predominantly in a particulate fraction. The photoaffinity-labeled 30-kD polypeptide was not observed in extracts from a mutant of *C. reinhardtii* that is believed to be deficient in an intracellular form of CA. These results provide evidence that the 30-kD polypeptide, which is photoaffinity labeled in lysed *C. reinhardtii* cells, is an intracellular form of CA.

In the unicellular green alga *Chlamydomonas reinhardtii*, CA is a component of an efficient system for the acquisition and accumulation of inorganic carbon for photosynthesis, which allows photosynthetic rates that are not limited by the low levels of CO_2 present in air (Aizawa and Miyachi, 1986; Sültemeyer et al., 1993). The predominant form of CA in this alga is a periplasmic enzyme that has been purified and extensively characterized (Yang el al., 1985; Kamo et al., 1990; Ishida et al., 1993) and is inducible when cells are transferred from growth under high CO_2 conditions to growth on air levels of CO_2 (Coleman et al., 1984). There is also a low level of a different periplasmic form of the enzyme present when cells are grown under high CO_2, and this enzyme has also been isolated (Rawat and Moroney, 1991). It is believed that periplasmic CA accelerates the extracellular equilibration of CO_2 and HCO_3^- to provide CO_2 at a sufficient rate to enter the cell and to serve as a substrate for photosynthetic reduction (Moroney et al., 1985). The periplasmic enzymes are particularly important for the acquisition of inorganic carbon at alkaline pH values where HCO_3^- is the predominant form of inorganic carbon in the medium (Moroney et al., 1985).

There is considerable evidence that an intracellular CA is also required for the efficient utilization of inorganic carbon. In a wall-less mutant of *C. reinhardtii*, the periplasmic CA is released into the growth medium (Kimpel et al., 1983), yet a low level of CA activity is observed upon cell lysis. This apparently intracellular CA activity is several orders of magnitude less sensitive to inhibition by aromatic sulfonamides than is the periplasmic enzyme (Husic et al., 1988; Sültemeyer et al., 1990). Furthermore, mutants of *C. reinhardtii* have been isolated and characterized that are deficient in the low intracellular CA catalytic activity. Although the mutants accumulate inorganic carbon to high levels within the cell, they are ineffective at the utilization of the intracellular inorganic carbon pools for photosynthesis (Spalding et al., 1983, 1985; Moroney et al., 1986; Katzman et al., 1989). Several studies indicate that intracellular CA is localized within the chloroplast (Katzman et al., 1989; Sültemeyer et al., 1990), suggesting that its role may be to accelerate the dehydration of HCO_3^-, which may be pumped into the chloroplast by an active transport mechanism, to provide CO_2 as a substrate for Rubisco (Moroney et al., 1987a; Sültemeyer et al., 1988; Moroney and Mason, 1991). Despite the evidence for intracellular CA activity in *C. reinhardtii* cells, the enzyme has not been specifically identified or isolated.

A variety of aromatic sulfonamides are known to be high-affinity, specific inhibitors of CA from a variety of sources (Maren, 1967), including from the alga *C. reinhardtii* (Husic, 1991). In this report we describe the synthesis of a CA-directed photoaffinity reagent formed by coupling a sulfonamide inhibitor of CA, PAMBS, to an aryl-azide group that is susceptible to radioiodination and photoactivation. The reagent was used to photoaffinity label isolated *C. reinhardtii* periplasmic CA and *C. reinhardtii* cell lysates to identify intracellular form(s) of the enzyme.

MATERIALS AND METHODS

Synthesis of PAMBS-ASA

Azido-containing reagents were handled in very dim, indirect light. PAMBS-HCl (9.1 μmol; Sigma) was dissolved in 0.50 mL of dimethylformamide (Aldrich), and 12.6 μL of

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Abbreviations: CA, carbonic anhydrase; I_{50}, concentration of an inhibitor that causes a 50% reduction in the catalytic activity of a given amount of an enzyme; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; PAMBS, *p*-aminomethylbenzenesulfonamide; PAMBS-ASA, *p*-aminomethylbenzenesulfonamide-4-azidosalicylamide.

triethylamine (9.1 μmol, Aldrich) was added followed by 9.1 μmol of NHS-ASA (Pierce). The mixture was stirred in the dark for 14 h at room temperature in a capped 1.5-mL vial. The reaction mixture was stirred into 10 mL of ice cold H_2O, the fine precipitate was collected by centrifugation, the supernatant was decanted, and the precipitate was dried overnight in vacuo. The residue was dissolved in 1.5 mL of CH_2Cl_2 and dried over 1 g of Na_2SO_4, and the CH_2Cl_2 was evaporated to dryness under a stream of N_2. The solid product was stored desiccated at −20°C in the dark. The elemental analysis of the product was completed by Atlantic Microlab (Norcross, GA). ^{1}H-NMR spectroscopy of 20 mM PAMBS-ASA in DMSO-d_6 (Aldrich) was carried out with a Bruker 300 MHz NMR, and chemical shifts were determined relative to tetramethylsilane (Aldrich).

To evaluate the photosensitivity of the product, 50 μM PAMBS-ASA in 20 mM K phosphate (pH 7.4) were placed in a quartz cuvette, and the solution was flashed with a Sunpak Auto622 photographic electronic flash unit placed at a distance of 1 cm from the cuvette. Absorption spectra were collected with a Hewlett-Packard diode array spectrophotometer the indicated number of flashes.

Radioiodination of PAMBS-ASA

PAMBS-ASA (100 nmol in 10 μL of dimethylformamide) was added to 100 μL of 0.1 M Na phosphate (pH 7.4), followed by 5.0 μL of 40 mM carrier-free Na^{125}I (0.5 mCi) (Dupont/NEN). The iodination reaction was initiated by the addition of 30 μL of 7.3 mM Chloramine-T (Aldrich) in 0.1 M Na phosphate (pH 7.4) and terminated after 15 min at room temperature by the addition of 100 μL of 26 mM $Na_2S_2O_5$ (Sigma). The reaction mixture was extracted three times with 1.0 mL of CH_2Cl_2, and the combined CH_2Cl_2 layers were dried over 5 g of Na_2SO_4, evaporated to dryness under a stream of N_2, dissolved in 100 μL of dimethylformamide, and stored in a desiccator in the dark at −20°C. No detectable product was retained in the aqueous layer following the triplicate extraction with CH_2Cl_2 (as judged by TLC), and the concentration of the final product in solution in dimethylformamide was estimated assuming 100% recovery. The reagent retained chemical stability (as judged by specific photoaffinity labeling of CA) for at least 6 months under these conditions.

Growth of *Chlamydomonas reinhardtii* Cells and Preparation of Cell Extracts for Photoaffinity Labeling

Chlamydomonas reinhardtii cells were grown in minimal medium (Sueoka, 1960) bubbled with either air (designated air grown) or air supplemented with 5% CO_2 (designated high CO_2 grown). The cell suspensions were maintained at 21°C with moderate shaking under continuous illumination with light supplied from cool-white fluorescent tubes. The *cia-3* mutant cells were high CO_2 grown and were switched to bubbling with air 30 h prior to the harvesting of cells. Wild-type *C. reinhardtii* (strain 137$^+$) was obtained from Dr. Robert Togasaki (Indiana University, Bloomington, IN), and the CA-deficient mutant *C. reinhardtii* strain *cia-3* was provided by Dr. James Moroney (Louisiana State University, Baton Rouge, LA).

Cells were harvested in the late log phase of growth by centrifugation at 3000*g* and then washed with ice-cold minimal medium and recentrifuged. Cells were resuspended in 25 mM Hepes-NaOH (pH 7.4), 0.5 mM PMSF (Sigma), and 0.1 mM DTT (Bio-Rad) to provide a cell suspension with a Chl concentration of 0.5 mg mL^{-1} for use in photoderivatization experiments. Except for those experiments involving intact cells, crude cell lysates were produced by two passes through a chilled Parr cell disruption bomb after equilibration of the cells for 5 min at 1500 p.s.i. Where indicated, the lysates were centrifuged at 12,000*g* for 20 min, the supernatant fraction was removed, and the pellet (particulate) fraction was resuspended in 25 mM Hepes-NaOH (pH 7.4), 0.5 mM PMSF, and 0.1 mM DTT to the same volume as the original lysate. These fractions were used directly for photoaffinity-labeling experiments.

Isolation of *C. reinhardtii* CA and Measurement of Catalytic Activity and Inhibition

Periplasmic CA was isolated from air-grown *C. reinhardtii* cells (strain 137$^+$) using PAMBS-agarose affinity chromatography by the method of Yang et al. (1985) as modified and described previously (Husic, 1991). CA catalytic activity was measured electrometrically by the method of Wilbur and Anderson (1948) as modified and described previously (Cohen and Husic, 1991). I_{50} measurements of isolated periplasmic CA by inhibitors was determined in CA assays containing 0.05 μg mL^{-1} (1.4×10^{-9} M active sites) of CA, activity was measured in the presence of inhibitor concentrations in the range of 0.1 to 100 μM, and the I_{50} values were estimated graphically. I_{50} values for inhibition by azido-containing reagents were measured in very dim, indirect light to minimize the possibility of irreversible inhibition.

Photoaffinity Labeling

Aliquots (10 μL) of each sample to be derivatized with ^{125}I-PAMBS-ASA were placed into separate wells of a Falcon 3911 microtiter plate, and 25 mM Hepes-NaOH (pH 7.4) was added to a total volume of 49 μL for a sample well and 48 μL for a control well. One microliter of 0.1 M ethoxyzolamide in 25 mM Hepes-NaOH (pH 10) was added to the control well. The sample containing ethoxyzolamide served as a control to evaluate the extent of nonspecific labeling of samples. ^{125}I-PAMBS-ASA (1 μL) in dimethylformamide was added to each well to obtain a final concentration of either 0.5 or 1.0 μM as indicated. The samples were immediately flashed 10 times with a Sunpak Auto622 photographic electronic flash unit from a distance of about 1 cm. Although fewer flashes were normally required for complete photoactivation of ^{125}I-PAMBS-ASA in aqueous solution, as many as 10 flashes were required for complete derivatization of proteins in biological samples containing other light-absorbing components. The reactions were quenched by the addition of SDS-PAGE sample buffer containing 10% β-mercaptoethanol (Sigma), after which the samples were placed in a boiling water bath for 1 min and bromphenol blue in 50% glycerol (15 μL of 0.01% [w/v]) was added prior to electrophoresis. In some control experiments, the reactions were quenched prior to illumination.

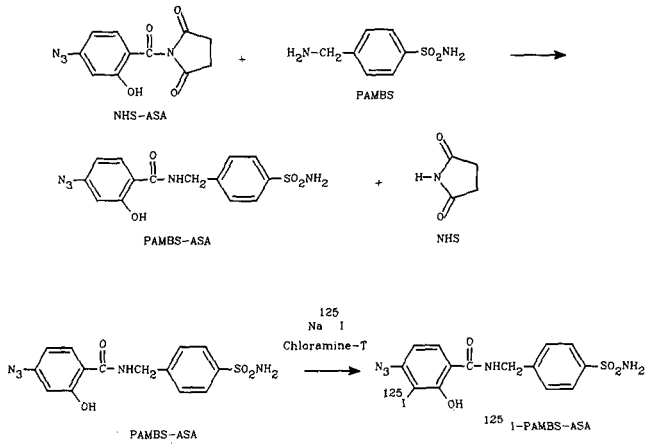

Figure 1. Scheme for the synthesis of ^{125}I-PAMBS-ASA.

Samples (20 μL) were subjected to SDS-PAGE with either 12 or 15% polyacrylamide (Bio-Rad) gels as indicated, using the buffer system of Laemmli (1970). Twenty percent polyacrylamide gels and the buffer system of Giulian et al. (1985) were used for the resolution of the 4-kD subunit of CA following the derivatization of purified periplasmic CA. In most experiments, electrophoresis was continued until the marker dye (and free ^{125}I-PAMBS-ASA) had just eluted from the bottom of the gel. In some experiments in which the dye was retained on the gel, free ^{125}I-PAMBS-ASA was observed as a dark spot at the bottom of the gel following autoradiography. Gels were fixed overnight with 1% glutaraldehyde (Sigma) (20% gels only), stained with Coomassie brilliant blue (BRL), destained, dried, and autoradiographed by exposure to Kodak X-Omat-AR film with Fisher Biotech intensifying screens at −80°C.

RESULTS

Synthesis and Chemical Characterization of ^{125}I-PAMBS-ASA

^{125}I-PAMBS-ASA was synthesized as summarized in Figure 1. The coupling of PAMBS and ASA resulted in the formation of PAMBS-ASA in 45% yield. The melting point of PAMBS-ASA was 218 to 220°C, and the R_F value of the product following TLC on silica layers developed with CH_2Cl_2:methanol (9:1, v/v) was 0.62. The results of the elemental analysis of the product revealed reasonable agreement between observed and predicted (in parentheses) values for the monohydrate; carbon, 46.43% (46.02%); hydrogen, 3.66% (3.59%); nitrogen 18.53% (19.17%). ^{1}H-NMR spectroscopy of PAMBS-ASA revealed the following chemical shifts: δ 7.97 ppm (d, 1H), 7.80 (d, 2H), 7.50 (d, 2H), 7.35 (s, 1H), 6.70 (dd, 1H), 6.60 (d, 1H), 4.59 (m, 2H), 3.35, 3.33 (2s, 2H), 2.50 (m, 1H). The subsequent radioiodination of PAMBS-ASA resulted in the incorporation of 0.83 mol of ^{125}I per mol of PAMBS-ASA.

PAMBS-ASA was judged to have retained the photoactivatable azido group based on the nitrogen content following elemental analysis of the product and by the light-induced decrease in the UV absorption characteristic of the azido group of ^{125}I-PAMBS-ASA (Fig. 2). Light is believed to activate the azido group to form a short-lived nitrene, which, free in aqueous solution, reacts with the solvent and results in diminished UV absorption (Bayley and Knowles, 1977). When photoactivation occurs after the reagent has bound to a protein, the nitrene is believed to add primarily to either C—H or O—H bonds to form a stable covalent adduct (Bayley and Knowles, 1977). The decrease in UV absorption attributed to the photolysis of the azido group shown in Figure 2 is similar to the results obtained by others with photoaffinity reagents prepared by the modification of other amine-containing enzyme inhibitors with NHS-ASA (Shanahan et al., 1985; van der Horst et al., 1990). The results in Figure 2 indicate that three flashes of light resulted in effective photolysis of the azido group, since there was little additional decrease in the UV absorption caused by additional flashes.

^{125}I-PAMBS-ASA was an effective inhibitor of purified periplasmic CA from *C. reinhardtii* with an I_{50} value of 1 μM. Similar I_{50} values were observed for inhibition of the enzyme by unlabeled PAMBS-ASA (I_{50} = 1 μM) and by PAMBS, the CA inhibitor derivatized to form the photoaffinity reagent (I_{50} = 0.9 μM).

Photoaffinity Labeling of Periplasmic CA

The labeling of CA by ^{125}I-PAMBS-ASA was demonstrated by the photoaffinity labeling of isolated periplasmic CA from *C. reinhardtii*. The reagent specifically modified the 37-kD large subunit of the enzyme; no apparent labeling of the 4-kD small subunit polypeptide was observed (Fig. 3). Photoaffinity labeling was prevented by the presence of a large excess of ethoxyzolamide, a sulfonamide with a high affinity for the periplasmic CA in *C. reinhardtii* (Husic et al., 1988), indicating that the labeling was specifically due to interaction with the sulfonamide-binding site of the enzyme. The specific labeling of the periplasmic CA was also observed upon the

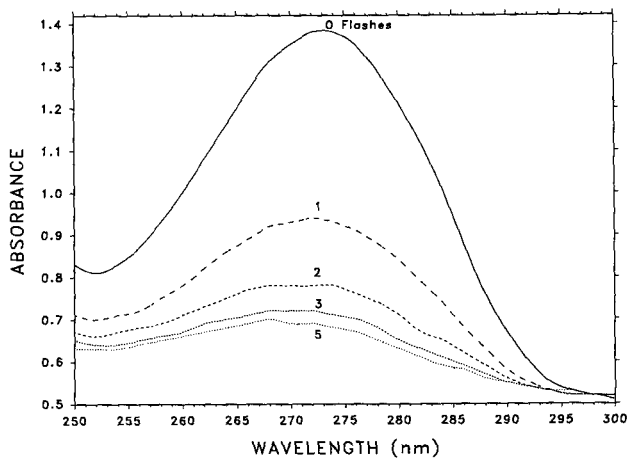

Figure 2. Photosensitivity of PAMBS-ASA. PAMBS-ASA (50 μM) in 20 mM K phosphate (pH 7.4) was flashed with light the number of times indicated, and absorption spectra were collected as described in "Materials and Methods."

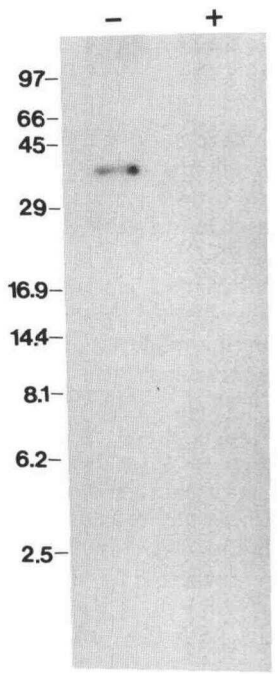

Figure 3. Photoaffinity labeling of isolated *C. reinhardtii* periplasmic CA. Autoradiograph following SDS-PAGE (20% polyacrylamide) of isolated periplasmic CA exposed to 1 μM ^{125}I-PAMBS-ASA and flashed 10 times with an electronic flash, either in the absence (−) or presence (+) of 2 mM ethoxyzolamide. Molecular mass values (kD) are indicated on the left.

treatment of intact cells of *C. reinhardtii* with photoactivated ^{125}I-PAMBS-ASA; the 37-kD polypeptide characteristic of the periplasmic CA large subunit was the only specifically labeled polypeptide (Fig. 4).

Photoaffinity Labeling of *C. reinhardtii* Cellular Lysates from Wild-Type Cells and from the CA-Deficient Mutant *cia-3*

Photoaffinity labeling of lysates from air-grown *C. reinhardtii* wild-type (137$^+$) cells revealed the labeling of several polypeptides (Fig. 5, lane A, and Fig. 6, lane B). Specifically labeled polypeptides were considered to be those labeled by light-activated ^{125}I-PAMBS-ASA but not labeled in the presence of an excess of the competing sulfonamide, ethoxyzolamide, an inhibitor of intracellular CA activity in *C. reinhardtii* (Moroney et al., 1985; Husic et al., 1988; Sültemeyer et al., 1990). No polypeptides were labeled if the reaction was quenched with β-mercaptoethanol prior to the photoactivation (Fig. 5, lane D). The most prominent specifically labeled polypeptides had molecular mass values of 37 and 30 kD. The 37-kD polypeptide characteristic of the periplasmic CA was sometimes partially obscured by a nonspecifically labeled polypeptide of similar apparent molecular mass, although the extent of nonspecific labeling was variable in different experiments. When cell lysates were centrifuged prior to photoaffinity labeling, the 30-kD photoaffinity-labeled peptide was localized predominantly in the particulate fraction, whereas the 37-kD polypeptide was observed in the soluble fraction (Fig. 5). The 30-kD labeled polypeptide was also observed in high CO_2-grown cells but in lower levels than in air-grown cells (Fig. 6).

When lysates of air-adapted cells from a mutant of *C. reinhardtii* deficient in intracellular CA activity (*cia-3*) were subjected to photoaffinity labeling, labeling of the 30-kD polypeptide was not observed, although the 37-kD polypeptide characteristic of the periplasmic CA was labeled (Fig. 6). This result is consistent with the suggestion that the 30-kD polypeptide specifically labeled by ^{125}I-PAMBS-ASA in *C. reinhardtii* wild-type cells represents an intracellular form of CA.

DISCUSSION

A CA-directed photoaffinity reagent (^{125}I-PAMBS-ASA) was synthesized that has a high affinity for periplasmic CA from *C. reinhardtii* and specifically covalently modifies the enzyme following photoactivation. The reagent also specifically modifies bovine CA (data not shown) and may be of value in the identification of forms of CA in variety of experimental systems. Although there are aromatic sulfonamides with higher affinities for CA, PAMBS was chosen for derivatization because it contains a reactive alkylamino group

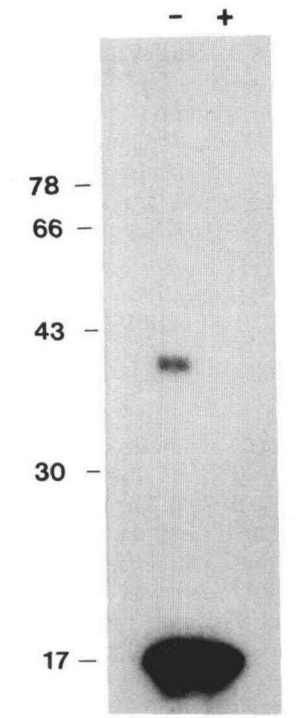

Figure 4. Photoaffinity labeling of intact *C. reinhardtii* cells. Autoradiograph following SDS-PAGE (12% polyacrylamide) of intact, air-grown *C. reinhardtii* cells exposed to 0.5 μM ^{125}I-PAMBS-ASA and flashed with light, either in the absence (−) or presence (+) of 2 mM ethoxyzolamide as described in "Materials and Methods." The dark band at the bottom of the gel represents free ^{125}I-PAMBS-ASA, which was not run off the end of the gel in this experiment as it was in the other experiments reported here. Molecular mass values (kD) are indicated on the left.

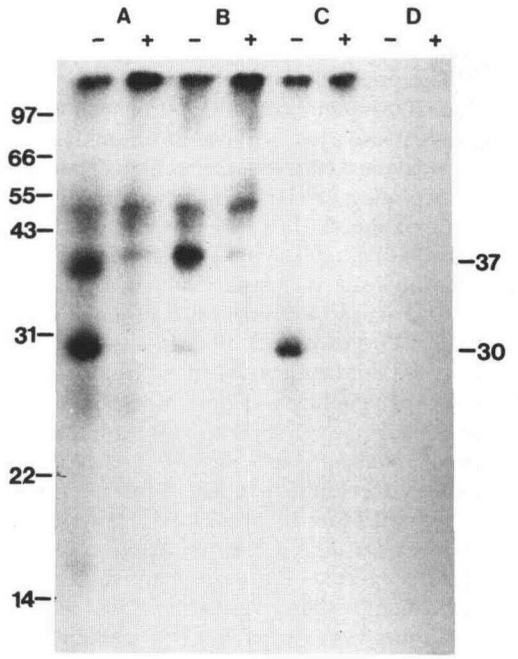

Figure 5. Photoaffinity labeling of lysed and fractionated *C. reinhardtii* cells. Autoradiography was carried out following SDS-PAGE (15% polyacrylamide) of lysed, air-grown *C. reinhardtii* cells exposed to 0.5 μM ^{125}I-PAMBS-ASA and flashed with light. Cell fractions were crude lysates (lanes A and D) or soluble (lane B) or particulate (lane C) fractions prepared as described in "Materials and Methods." The sample in lane D was a control in which the reaction was quenched by the addition of SDS-PAGE sample buffer containing β-mercaptoethanol prior to illumination. Samples in the lanes designated + contained 2 mM ethoxyzolamide during photoaffinity labeling. The migrations of molecular mass markers (kD) are shown on the left, and the estimated molecular mass values for specifically labeled polypeptides are shown on the right.

that promotes effective coupling with NHS-ASA. Affinity labeling of CA has been previously reported using [^{14}C]-bromoacetazolamide (Kandel et al., 1968) and either ^{3}H- or ^{35}S-labeled *p*-azidobenzenesulfonamide (Hixson et al., 1980); however, ^{125}I-PAMBS-ASA combines the desirable features of control of the reaction by photoactivation, a rapid rate of reaction, and the benefit of the high sensitivity for the detection of the ^{125}I label.

Kamo et al. (1990) demonstrated that *C. reinhardtii* periplasmic CA contains both 37- and 4-kD subunits; however, when ^{125}I-PAMBS-ASA was utilized for the photoaffinity labeling of isolated CA, only the large (37 kD) subunit was modified (Fig. 3), despite the suggestion that at least part of the small subunit may be close to the active site Zn^{2+} responsible for sulfonamide binding (Fukuzawa et al., 1990). When intact *C. reinhardtii* cells were photoaffinity labeled with the ^{125}I-PAMBS-ASA, the 37-kD polypeptide characteristic of the predominant form of periplasmic CA was observed to be specifically labeled (Fig. 4).

When cells were lysed and fractionated by centrifugation prior to photoaffinity labeling with ^{125}I-PAMBS-ASA, a prominent specifically labeled 30-kD polypeptide was observed in the particulate fraction (Fig. 5). The protection of labeling of the 30-kD polypeptide by ethoxyzolamide is consistent with the possibility that this polypeptide represents an intracellular form of CA; however, this result alone does not preclude the possibility that this polypeptide is a sulfonamide-binding protein that is unrelated to CA.

To provide further evidence to support the identity of the 30-kD polypeptide as intracellular CA, the photoaffinity labeling of extracts from a CA-deficient mutant was carried out. The 30-kD polypeptide susceptible to specific photoaffinity labeling by ^{125}I-PAMBS-ASA was not detected in lysates from the *C. reinhardtii* mutant *cia-3* (Fig. 6), a mutant deficient in intracellular CA activity (Moroney et al., 1986; Katzman et al., 1989). The evidence that this mutant is deficient in intracellular CA includes measurement of decreased CA activities in isolated chloroplasts (Katzman et al., 1989) and the inability of the mutant to utilize accumulated intracellular pools of inorganic carbon for photosynthesis (Moroney et al., 1986). Spalding et al. (1991) demonstrated that the *C. reinhardtii* mutant *ca-1*, a CA-deficient mutant that is allelic to *cia-3*, displayed substantial pleiotropic reductions in a number of polypeptides. Thus, the reduction of the

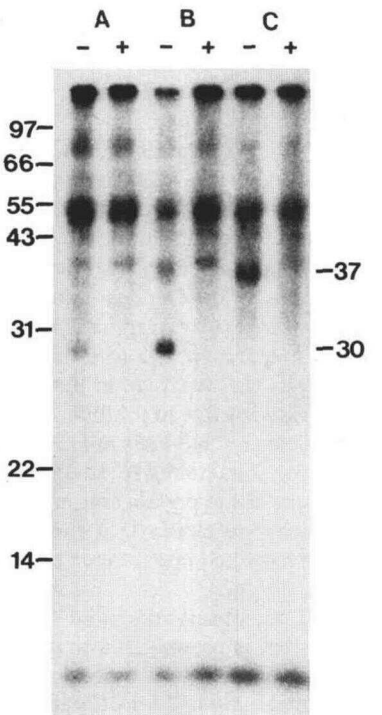

Figure 6. Photoaffinity labeling of lysed 137^{+} and *cia-3 C. reinhardtii* cells. Autoradiography was carried out following SDS-PAGE (15% polyacrylamide) of lysed *C. reinhardtii* cells exposed to 0.5 μM ^{125}I-PAMBS-ASA and flashed with light. Cell lysates were obtained from CO_2-grown wild-type (137^{+}) cells (lane A), air-adapted wild-type (137^{+}) cells (lane B), and air-adapted *cia-3* cells (lane C), as described in "Materials and Methods." Samples in the lanes designated + contained 2 mM ethoxyzolamide during photoaffinity labeling. The migrations of molecular mass markers (kD) are shown on the left, and the estimated molecular mass values for specifically labeled polypeptides are shown on the right.

30-kD polypeptide in *cia-3* cannot be definitively ascribed to the primary genetic lesion in the mutant. However, the combined evidence of protection of photoaffinity labeling of the 30-kD polypeptide by ethoxyzolamide and the absence of the polypeptide in the *cia-3* mutant cells strongly implicates the 30-kD polypeptide as an intracellular CA. Furthermore, the presence of the polypeptide in a particulate fraction and its presence in decreased levels in high CO_2-grown cells compared to air-grown cells are also consistent with prior studies based on activity measurements of intracellular CA (Husic et al., 1988; Katzman et al., 1989; Sültemeyer et al., 1990).

The prominence of the 30-kD photoaffinity-labeled polypeptide compared to the 37-kD periplasmic CA was unexpected. Although we have not yet determined the reasons for this observation, several possible explanations can be considered. It is possible that the abundance of intracellular CA may be greater than the very low catalytic activities measured previously (Husic et al, 1988; Katzman et al., 1989; Sültemeyer et al., 1990) as a result of partial catalytic inactivation associated with cell lysis. We have observed previously with the periplasmic CA from *C. reinhardtii* that it is possible for sulfonamide binding to be retained, even after inactivation of catalytic competence (Husic et al., 1991). Alternatively, the low apparent catalytic activity of intracellular CA may result from an inherently low catalytic turnover rate for the enzyme. In addition, the labeling intensities of the 37- and 30-kD polypeptides may also reflect differences in the binding affinities of these two proteins for ^{125}I-PAMBS-ASA. In the photoaffinity-labeling experiments reported here, the concentration of ^{125}I-PAMBS-ASA used (0.5 μM) was less than saturating with respect to binding to the periplasmic CA (I_{50} = 1 μM). This was necessary to minimize the nonspecific labeling of proteins in the cell extracts. Therefore, differences in the binding affinities of different proteins for ^{125}I-PAMBS-ASA make it impossible to extrapolate from the labeling intensity of different polypeptides to estimate their relative abundance. However, the observation that the intracellular CA appears to be less sensitive to inhibition by sulfonamides than the periplasmic enzyme (Husic et al., 1988; Sültemeyer et al., 1990) implies that its intensity should appear less than a comparable amount of the periplasmic enzyme. Additional work will be necessary to clarify that those factors are responsible for the unexpected apparent abundance of the 30-kD polypeptide.

As noted above, our observation that the 30-kD photoaffinity-labeled protein is present in a particulate fraction is consistent with prior observations based on catalytic activity measurements of intracellular CA in *C. reinhardtii* (Katzman et al., 1989; Sültemeyer et al., 1990). The insolubility of the 30-kD protein may explain why the protein has not been identified previously by those using PAMBS-agarose affinity chromatography of extracts of soluble proteins from lysed *C. reinhardtii* to isolate the periplasmic CA. These results suggest that the 30-kD protein is membrane associated or tightly associated with another insoluble cellular component. Additional work will be necessary to establish the specific localization of the intracellular protein in *C. reinhardtii* cells.

A number of components of the inorganic carbon-concentrating system in *C. reinhardtii* are present when cells are grown under CO_2-limiting conditions (air levels of CO_2) but are absent when cells are grown under high CO_2 conditions (5% CO_2). The predominant periplasmic form of CA is induced under CO_2-limiting conditions (Coleman et al., 1984); however, there is also a low activity of a different extracellular form of the enzyme that is present in high CO_2-grown cells (Rawat and Moroney, 1991). Direct activity measurements in lysed cells (Spalding et al., 1985; Husic et al., 1988; Sültemeyer et al., 1990) as well as physiological studies (Moroney et al., 1987b) indicate that intracellular CA is also present in both air- and high CO_2-grown cells, although its levels are lower in high CO_2-grown cells than in air-grown cells. The results presented here are consistent with those studies; the labeled 30-kD polypeptide was present in both air and high CO_2-grown cells, although its level was significantly greater in air-grown *C. reinhardtii* cells (Fig. 6).

A preliminary accounting of some of this work (Husic and Marcus, 1993) noted that 26- and 21.5-kD polypeptides were specifically labeled using ^{125}I-PAMBS-ASA; however, the 30-kD polypeptide reported here was not apparent. Those studies were carried out with detergent-solubilized cells, whereas the present studies were carried out with pressure-lysed cells in the presence of the protease inhibitor PMSF and a low concentration of DTT. Preliminary studies indicate that the 30-kD polypeptide labeling is prevented by the presence of 3-(3-chloramidopropyl)dimethylammonio-1-propanesulfonate, the detergent utilized in the prior studies (data not shown). Although a specifically labeled soluble 26-kD polypeptide was sometimes observed in experiments carried out as described here when autoradiograms were subjected to longer exposure times (data not shown), the 21.5-kD polypeptide reported previously was not observed. The protocol reported here reduces the extent of nonspecific labeling and does not cause inactivation of the 30-kD polypeptide with respect to its ability to bind ^{125}I-PAMBS-ASA. The results presented here support the conclusion that the 30-kD polypeptide represents intracellular (chloroplastic) CA; however, further studies will be required to establish whether the 26- or 21.5-kD polypeptides represent other intracellular forms of CA in *C. reinhardtii*.

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